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Progress Report
of Work Done under Navy Contract N6-ONR-26007

from JAN 1 1952

to JUN 30 1952

Respectfully submitted by:

Lera M. Deans

Principal Investigator

Arthur W. Davidson

Assistant Dean, University of
Kansas Graduate School.

TABLE OF CONTENTS

	Page
Summary Semi-Annual Report, July 1, 1951 to December 31, 1951	1
Summary Thesis Joan McCamish, 1952	3
Summary Semi-Annual Report, January 1, 1952 to June 30, 1952	4
Outline of Report	6
Personnel	8
Materials and Methods	10
Technical Report:	
Antigen Studies	13
Immunity Studies:	
A. Protection Tests	17
B. Bactericidal Tests.....	26
C. Ascoli Tests	33
D. Mouse Clearance Tests	43
Variation Studies:	
A. Antigenic response to variant strains in rabbits and rats....	53
B. The behavior of smooth virulent forms in white mice and rats.	61
C. The multiplication of variants in white mice	65
Discussion	67

TABLES AND FIGURES

	Page
Table 1 The immunogenic effect of tissue extracts from infected mice ...	16
Table 2 Summary of the protective effect for white mice of serum from vaccinated and recovered rabbits	20
Table 3 The protective effect of serum from rabbits after recovery from a living dose of the variant strains of Jap	21
Table 4 The protective effect of serum from rabbits after recovery from two living doses of the variant strains of Jap	22
Table 5 Summary of the protective effect for white mice of whole serum and of fractions from recovered rabbits	25
Table 6 Bactericidal effect of human blood	27
Table 7 Bactericidal effect of human blood	28
Table 8 Bactericidal effect of rabbit blood	29
Table 9 Bactericidal effect of rabbit blood	30
Figure 1	31
Figure 2	32
Table 10 The number of mice showing the Ascoli antigen following death from challenge	34
Table 11 Development of the Ascoli antigen in mice	36
Table 12 Pricipitin tests and Ascoli antigen extracts from cultures of variant strains	39
Table 13 Summary of the results of the injection of tissue extracts prepared by Larsons method	42
Figure 3	45
Figure 4	46
Table 14 Clearance tests in mice. Average of five typical experiments in actively immunized mice	48
Figure 5	50
Figure 6	52
Table 15 Comparison of the reciprocal of the average agglutinin titers in rabbits and rats	56

TABLES AND FIGURES (Cont.)

	Page
Figure 7	57
Figure 8	58
Table 16 Comparison of average agglutinin titer with average day of death following challenge with Sm A	60
Table 17 The immunogenicity of Sml in mice treated with dihydro-streptomycin	63
Table 18 The multiplication and morphology of variants during infection in mice	66

STUDIES ON THE PATHOGENESIS AND IMMUNITY OF TULAREMIA

SUMMARY

July 1, 1951 through December 31, 1951

Contract N6-ONR-26007
University of Kansas, Lawrence

The experiments which are summarized fall into 3 general categories:

I. Antigen Studies, II. Immunity Studies and III. Variation Studies.

I. Antigen Studies

1. Progress has been made in growing large quantities of Bacterium tularensis by using concentrated media in a cellophane sack suspended in distilled water. The cells have been used for the extraction of polysaccharide and are to be used for other types of extraction.

2. Sterile tissue extracts from infected animals have been used in preliminary experiments and have indicated the presence of an immunizing antigen.

II. Immunity Studies

3. A protection test in mice has been developed which appears to be reliable.

4. The results of protection tests appeared to show that the serum from recovered animals was more protective than from vaccinated animals.

5. There was no correlation between protective, agglutinative or hemagglutinative antibodies.

6. A satisfactory bactericidal test has been worked out.

7. This test indicates that whole blood from rabbits vaccinated with killed cultures is more bactericidal than blood from normal rabbits.

8. Blood from recovered or recently vaccinated persons is more bactericidal than blood from normal persons or those vaccinated a number of months before the test. Bacteriostatic effect might be a better term than bactericidal, since the blood is rarely sterilized and only small numbers of bacteria are disposed of.

III. Variation Studies

9. Variants of the partially virulent Jap strain are shown to vary in toxicity for white mice when injected in large numbers in the living state.

10. Variants of the Jap strain have been shown to vary in antigenicity as tested by the agglutination test after injection into rabbits. The smooth strain showed a greater degree of antigenicity than the non-smooth strain. This was in agreement with the findings reported earlier in white mice.

IMMUNOLOGICAL STUDIES ON TULAREMIA

by
Joan McCamish

Summary and Conclusions

1. A protective test, using immune rabbit serum and strain Ince of Bacterium tularensis in the susceptible white mouse, has been developed and the procedure for this test has been presented.

2. This test is useful in detecting the presence of and measuring the relative amounts of protective antibody in a tularemia antiserum.

3. A noticeable difference was found in the protective power of sera from rabbits following vaccination and from those following infection. The sera from convalescent rabbits offered protection approximately one hundred times that of sera from vaccinated rabbits.

4. At the dilutions of serum used, no differences could be observed in the protective capacity of several rabbit sera after sub-lethal infection with variants of strain Jap of Bacterium tularensis, despite the differences in virulence and immunogenicity of the variants for white mice.

5. The injection of sensitized red blood cells into a normal rabbit elicits the production of hemagglutinins as well as a low titer of the protective antibody and bacterial agglutinins.

6. No correlation has been found between the results of the protective test and the results of either the hemagglutination or the agglutination test.

-4-

SUMMARY

Antigenic Studies:

1. Polysaccharides from a virulent and partially virulent strain have been prepared and partially characterized.
2. These polysaccharides are not immunogenic for white mice.
3. Antigens isolated from cold macerated tissues from infected mice prolong life when injected into normal mice, but do not enable them to resist challenge with virulent organisms.

Immunity Studies:

1. A protective test has been worked out and evaluated.
2. The protective power of rabbit serum for mice has been shown to be greater from recovered rabbits than from vaccinated rabbits.
3. Bactericidal tests on human and rabbit whole blood have shown that blood from recovered humans and rabbits is more bacteriostatic than from vaccinated humans or rabbits.
4. A series of tests on Ascoli antigens are reported. These tests have shown that the antigen appears in mice as soon as abundant proliferation of the organism occurs and is generally lacking in challenged immune animals.
5. The Ascoli antigen apparently is not immunogenic for white mice.
6. Clearance tests on white mice show that the actively or passively immune mouse removes living cells from the blood stream with great rapidity and concentrates them in the liver. The implications of this mechanism are discussed.

Variation Studies:

1. A summary of the work on variants shows that the smooth and non-smooth strains can be divided into two distinct groups on the basis of their respective properties.

2. Experiments are cited which show that non-smooth strains do not produce as high a titer of agglutinins in rabbits as the smooth strains and neither is immunogenic.

3. The non-smooth strains produce a lower titer of agglutinins in rats than the smooth strains but are immunogenic.

4. No in vivo dissociation of smooth strains has been observed in normal or immune rats or mice.

Outline for
OFFICE OF NAVAL RESEARCH
Microbiology Branch

SEMI-ANNUAL PROGRESS REPORT

Report prepared by: Dr. Cora M. Downs

Date: July 1, 1952
For Period February 1, 1952
to June 30, 1952

NR:

Contract: N6-ONR-26007

Annual Rate: \$20,928.30

Contractor: University of Kansas

Principal Investigator: Cora M. Downs

Assistants: Research Assistants. See Personnel.

Title of Project: Studies on the Pathogenesis and Immunity of Tularemia.

Objectives: The study of the mechanism of immunity in tularemia by means of an attempt to isolate an immunogenic antigen and to understand the role of circulating antibodies and tissue cells in the immune animal.

Abstract (or Summary of Results)

a. Since start of project:

b. During current report period:

Plans for Future:

Immediate: Continue a study of the immunity mechanisms of mice and rats with special emphasis on tissue reactions. Attempt to isolate an active antigen which is immunogenic as well as serologically active.

Long range: The problems of immunity in tularemia pose many intriguing questions. There is a great difference in host response as indicated by the solid immunity produced in white rats by killed vaccines and the almost complete lack of immunity in white mice, rabbits, guinea pigs, cotton rats and other animals induced by these same vaccines. The latter animals become immune to varying degrees when injected with living organisms of lowered virulence. This may indicate that the multiplying organisms in the infected animal produce an immunogenic antigen or that

the immunogenic antigen for the white mouse and other animals is highly labile and that rats and more resistant animals respond to other antigens available in killed cultures. The elucidation of this question of immunogenic antigen is one of the long range objectives of this project.

It has been abundantly proven in this laboratory that immune animals dispose of large numbers of virulent organisms in a few days after challenge. It has been shown also that the bacteriostatic or bactericidal effect of whole blood serves to dispose of only a few organisms, 400 to 4000 per ml. Preliminary studies on clearance of the blood stream of organisms in immune animals show that the liver played a major role in accumulating organisms and rendering the blood stream sterile. The interaction of circulating antibodies and phagocytic mechanisms are therefore being scrutinized.

Studies in this laboratory and at Camp Detrick have shown that dissociation occurs in Bacterium tularensis cultures and that immunogenicity, virulence, colony type, toxicity and ability to multiply in the animal body are characteristic of the variants. The correlation between these various properties is far from clear and is also being studied as part of the long range program.

SEMI-ANNUAL REPORT CONTRACT N6-ONR-26007

The following report constitutes an account of the work done during the period from January 1, 1952 through June 30, 1952, together with a brief summary of this work, a summary of Miss Joan McCamish's thesis and a summary of the work done from July 1, 1951 through December 31, 1951.

Routine matters such as virulence titrations, the preparation of vaccines, etc., are not reported. Detailed reports on work now in progress are not included. The routine technics are given under Materials and Methods and any special technics needed to explain the experimental work are included with the report of the experiments.

PERSOMNEL

Contract N6-ONR-26007 was activated July 1, 1951. The following personnel is listed together with their status and time of employment.

Graduate Student Research Assistants

Employed

Max Moody, Ph.D. candidate

July 1, 1951, 1/2 time

Lloyd Hendrix, Ph.D. candidate

July 1, 1951, 1/2 time

Joan McCamish, MA candidate

July 1, 1951, 1/2 time
to May 31, 1952

Miss McCamish received her MA, June 1952 and will go to work for the Naval Laboratory, Berkeley California, September 1952.

Technicians

Mrs. Mary Jane Taylor, MA Bacteriology

July 1, 1951, full time

Mrs. Irene Dyke, L'ecole Rachel, Paris
Lycee Brizeux, Quimper

Oct. 1, 1951, full time

Patricia Brown, Secretary

Sept. 1, 1951, 1/2 time

Animal Care

Mr. Harry Jeffrey

July 1, 1951, full time

Notes: Mr. Moody will be with the project until January 1953, when he will receive his Ph.D.

Mr. Hendrix received his Masters degree in June 1952, and is continuing work on a Ph.D.

We expect to appoint one or more graduate students to the project in September 1952.

MATERIALS AND METHODS

The following is a description of general materials and methods used. Whenever necessary, details are given with the separate experiments.

1. Glucose cysteine blood agar as described by Downs⁽¹⁾ is used for surface plate counts and for slants for carrying stock cultures. (Hereafter designated as GCBA)

2. Snyders broth is usually used as described by Snyder⁽²⁾ for all liquid cultures.

3. A standard suspension is defined as a suspension of such turbidity as to give a light transmission of 24 in the Coleman spectrophotometer, 600 wave lengths. Such a suspension gives a plate count varying from 2 to 4×10^8 per ml. and is used as a starting suspension for all LD_{50} titrations so that an LD_{50} of 10^{-9} indicates that 1 to 2 organisms kill 50 per cent of the mice injected with 0.5 ml of the standard suspension. The Reed and Muench⁽³⁾ method is used to calculate the LD_{50} . A standard suspension is hereafter designated as (SS).

4. The usual stock strains used are Sm virulent strain LD_{50} for mice $10^{-8.5}$ (SS).

Strain 38 completely avirulent for mice.

Strain Jap partially virulent for mice LD_{50} $10^{-4.0}$.

Strain Ince partially virulent for mice LD_{50} $10^{-7.0}$.

In cases where variants are used, the history of the variant is given.

5. Albino mice of approximately 20 gram weight predominantly male are used. These mice are purchased from the Maple Grove Rabbitry, Springfield, Missouri, and are healthy vigorous stock.

6. All agglutination tests are performed using Strain 38, killed with phenol, washed and suspended in saline to standard turbidity. Equal

volumes of antigen and 2 fold dilutions of serum are mixed and incubated over night in the refrigerator for final reading the next day. The titre represents the highest dilution of serum at which definite agglutination may be seen with the naked eye and a concave mirror.

7. Hemagglutination tests were set up following the technique of Wright⁽⁴⁾.

Cells: 5 to 7 ml type O human blood was added to 1 ml 2 per cent potassium oxalate, the suspension washed 3 times in 0.85 per cent saline, and the cells packed and resuspended to 10 per cent in 0.85 per cent saline.

Carbohydrate: The lyophilized carbohydrate was made up in a solution of 1 mg/ml in 0.85 per cent saline and kept frozen.

Sensitization: The carbohydrate solution is diluted to .005 per cent with 0.85 per cent saline and added to an equal volume of 10 per cent erythrocytes. This mixture is incubated 4 hours in a 37° water bath. At the end of the incubation period the cells are washed four times in six or more volumes of 0.85 per cent saline and resuspended to .5 per cent in saline.

Agglutination: To 0.5 ml amounts of 2 fold serial dilutions of the serum, 0.5 ml of the 0.5 per cent cell suspension are added. The tubes are shaken and allowed to stand at room temperature for two hours. The test is read as the highest dilution of antiserum which shows 4+ agglutination (cells distributed uniformly over the bottom of the tube).

8. In the bactericidal tests, all glassware in contact with the heparinized blood was coated with silicone and chilled. Heparin was added so that the final dilution was 1 mg per 10 ml of blood. No difficulty with clotting during incubation was experienced.

9. Protection Tests: These tests were all performed in the following manner: Mice were given the immune or normal serum by the intravenous route, injecting 0.5 ml of the appropriate dilution into the tail vein. They were then immediately injected with the challenge strain Ince in dilutions such as to give 10 or 100 LD₅₀. The mice were held for 10-12 days and any dead mice were autopsied and the heart blood and spleen were cultured. Only specific deaths are recorded.

TECHNICAL REPORT

The technical report falls into three categories: I. Antigen Studies, II. Immunity Studies, III. Variation Studies.

I. ANTIGEN STUDIES

A. Preparations of Purified Antigens:

The method for the growth of large numbers of Bacterium tularens was given in the semi-annual report of December 31, 1951. At that time 7.8 grams of cells strain Sm, were on hand ready for extraction. These cells were used to prepare the polysaccharide and yielded 1.59 grams of material with a nitrogen value of 7.3 per cent. Since that time 8.0 grams of Jap H have been collected and are now being used to prepare another lot of polysaccharide. Cells of Sm and Jap are being grown to prepare protein extracts. The result of these experiments will be reported subsequently.

The injection of the Sm polysaccharide referred to above, gave evidence of very little immunogenic activity in mice. Out of 20 mice injected with a total of 1.0 mg in broken doses, 18 died when challenged with 100 LD₅₀ of the virulent Sm strain. Ten out of ten control mice died.

B. Tissue Antigens:

In the first progress report, results of the injection of tissue antigens prepared from mice injected with Jap H showed that they induced a prolongation of life in mice challenged with the fully virulent strain. Because of the great difficulty in ascertaining whether all of the living Jap H organisms which might have been present were killed by the freezing treatment, the experiment was repeated using tissues from mice injected with Jap H 0.5 ml of 10⁻⁴ intraperitoneally. A second experiment was also

set up, infecting mice with the virulent Sm strain. This strain has an LD₅₀ of $10^{-3.6}$ which represents 1 to 4 organisms. It was felt therefore that sterility of the preparations could be more easily established by the injection of the tissue into the control mice used to determine sterility than when using the Jap H strain.

The tissues were treated as described previously. Mice were infected with 1 ml of a standard suspension of Sm diluted to 10^{-7} . All mice were sacrificed at 24, 48, 72, and 96 hours after injection. The liver, spleen and heart were removed aseptically and 5 parts of chilled distilled water was added. The mixture was homogenized and centrifuged. The supernatant was removed and the material shall frozen and stored in the deep freeze. All the above operations were carried out as rapidly and in as low a temperature as possible. The detailed data on the sterility tests on the tissue is given in the appendix and may be summarized here by saying that tissue from Jap H infected mice remained infective for 21 days but not for 35 days. Whereas cultures were negative after 10 days. Tissue from Sm infected mice was infective for 45 days but not for 75 days and by culture they were positive for 10 days but not for 25 days.

The above tissue extracts were injected into groups of 18 mice 0.5 ml intraperitoneally on alternate days until 4 doses had been given. After a period of 10 days they were challenged with the virulent strain Sma. Normal control mice were given comparable tissue extracts prepared from normal tissues. Results of these two experiments are summarized in Table 1.

There is very slight evidence that an immunogenic antigen is present in these tissue extracts. There is slight lowering of the LD₅₀ and slight prolongation of life. The concentration of this hypothetical antigen will

be explored by chemical means. We have frequently reiterated our views that immunogenicity rests principally on the production of some antigen produced in the animal body during infection, but have been unable to get clear cut evidence of this antigen. That antigens are demonstrable from tissues is evident from the work of Larson⁽⁴⁾ and this laboratory⁽⁵⁾, but it is not clear that these heat stable antigens are responsible for immunity.

be explored by chemical means. We have frequently reiterated our views that immunogenicity rests principally on the production of some antigen produced in the animal body during infection, but have been unable to get clear cut evidence of this antigen. That antigens are demonstrable from tissues is evident from the work of Larson⁽⁴⁾ and this laboratory⁽⁵⁾, but it is not clear that these heat stable antigens are responsible for immunity.

Table 1
The immunogenic effect of tissue extracts from infected mice

Challenge Dose SMA	Tissue extract from Jap H infected mice--Days of sacrifice designated by sub-number. Normal controls received normal tissue.									
	T ₂₄					T ₇₂				
	Dead tested	ADD	LD ₅₀	Dead tested	ADD	LD ₅₀	Dead tested	ADD	LD ₅₀	Dead tested
10 ⁻⁷	6/6	5.3	3/6	10	6/6	3.6	5/6	4.8	6/6	4.5
10 ⁻⁸	4/6	5.8	1/6	10	5/6	7.4	6/6	6.0	6/6	4.3
10 ⁻⁹	4/6	6.5	10 ^{-9.0+}	0/6	1/6	5.0	5/6	6.6	10 ^{-9.0+}	5/6
10 ⁻¹⁰							0/6			0/6
										10 ^{-9.4}
Tissue extracts from Sm infected mice. Days of sacrifice were the same as above.										
10 ⁻⁷	6/6	4.7	6/6	5.0	6/6	6.0	4/5	6.8	5/5	4.6
10 ⁻⁸	6/6	5.0	4/6	5.0	6/6	8.3	6/6	7.1	6/6	5.5
10 ⁻⁹	5/6	6.0	3/6	5.7	4/6	8.5	3/6	7.0	6/6	5.0

The LD₅₀ of the challenge strain SMA 10^{-9.4} in both cases.

Immunity Studies

A. Protection Tests - Whole Rabbit Serum

In the first six months report we described the isolation and stabilization of the Ince strain of Bacterium tularensis. This strain has retained an LD₅₀ of approximately 10⁻⁷ and has been used in subsequent protection tests. In an effort to evolve some simple method of evaluating these tests we have used Pannell's⁽⁶⁾ method of calculation of the mouse protection index and mouse protection ratio. In addition, McCamish⁽⁷⁾ attempted to simplify the above method by eliminating the average day of death from the calculation and made a comparison on the basis of the number of mice dead at a given dilution of organisms after receiving normal serum and the number dead after receiving immune serum. The difference was thus a small whole number. The difference at any one level was designated as Δ. If the differences at different levels are added, the protective power of the serum could be designated as Sigma. Examples of these calculations are given as follows:

$\frac{\text{Per cent mortality}}{\text{Average day of death}} = \text{mouse protection index}$

Normal Serum $\frac{5/5 \text{ (dead over tested)}}{5 \text{ ADD}} = \frac{83.3}{5} = \text{MPI of } 16.7$

Immune Serum $\frac{1/5}{6} = \frac{20}{6} = \text{MPI of } 3.3$
 $\frac{\text{MPI } 16.7}{\text{MPI } 3.3} = \text{MPR of } 5.1$

The smaller the numerical value of MPI, the greater the protection.
 The larger the numerical value of MPR, the greater the protection.

$\Delta = \frac{\text{dead}}{\text{tested}} \text{ Normal serum } 1:10 + \text{ Ince Strain } 10^{-8} \quad \text{Minus} \quad \frac{\text{dead}}{\text{tested}} \text{ immune serum } + \text{ Ince } 10^{-8}$

thus: $\frac{5}{6} - \frac{1}{6} = \Delta 4$

$$\Delta = \begin{array}{l} \text{Normal serum 1-10} \\ + \text{Ince } 10^{-6} \end{array} \quad \text{minus} \quad \begin{array}{l} \text{Immune serum} \\ + \text{Ince } 10^{-6} \end{array}$$

$$\text{thus: } 6/6 - 0/6 = \Delta 6$$

$$\Sigma \text{ then is } \Delta^4 + \Delta^6 = \Sigma 10$$

The individual reactions of the mice to infection made evaluation of the sera on the simple basis of ascending numerical values of Σ impractical. In order to compare the sera quantitatively, it was necessary to establish some arbitrary standard as a protective level for Σ . The maximum value of 12 was not obtained in any case, but the value of 10 occurred most often in the case of the high values of Σ , half of that or 5 was then assumed to be the lowest value which a serum could have and still be considered protective. The protective titer of a serum would then be the highest dilution of that serum showing a value of 5 or above.

In the method used by Pannell a serum was assumed to be protective if the MPR was 2 or above. When the MPR of the sera used was compared to the corresponding Sigma it was found that in all but a few cases the results correlated. In only 3 cases was the MPR above the protective level of 2 when the Σ was less than 5.

The protection tests were performed as given in Materials and Methods. Three pools of serum were used from rabbits receiving phenolized vaccines, containing 4 billion organisms per ml. The vaccines were given intraperitoneally in 2 ml amounts for 5 doses at 2 week intervals. Pools 1 and 2 came from 2 rabbits bled after receiving one and 2 injections of vaccines respectively — Pool 5 from 2 rabbits after the 5th injection. Rabbit V₃ and V₅ were unpooled samples from a rabbit after 3 and 5 injections respectively. Rabbit IV and V received a living Jap suspension containing

40 million cells 3 months after the last dose of phenolized vaccine. The protective effect of these sera is given in Table 2.

A series of rabbits ~~were~~ infected with 400 million living cells of Jap variants and were bled 10 days after infection. After a rest period of a week they received a second dose of living cells and were again bled 7 days later. These results are given in Tables 3 and 4.

The following summary is made from these results.

When the various values were calculated as indicated above, pooled serum from rabbits vaccinated with phenolized Jap vaccines gave a Sigma value above 5 only when diluted 1 to 10, except in the case of one rabbit which had been given 5 series of vaccine injections. Serum from 2 vaccinated rabbits which had recovered from a challenge dose of 40 million Jap cells gave a Sigma value of 5 and 8 respectively at a dilution of 1:1000. Serum from rabbits injected with 400 million living cells of variant strains of Jap, that is Jap A, B, C, and H, and bled 10 days after the injection, gave Sigma values of 6, 10, 11, and 11 respectively. These same rabbits when allowed to rest and when given another challenge dose of equal quantity gave Sigma values of 10, 10, 10 and 9 respectively. It seems reasonable from these experiments to conclude that rabbit serum contains protective antibodies for the mouse which are measurable by this method.

2

Table 2
Summary of the protective effect for white mice
of serum from vaccinated and recovered rabbits

Source	Dilution	Immune			Normal				Σ
		Dead ★ tested	ADD	MPI	Dead tested	ADD	MPI	MPR	
Pool #1 Phenolized Vaccine	1:10	1/11	6.0	1.5	11/12	4.7	19.5	13	10
	1:1000	7/12	5.6	10.4	7/11	5.7	11.1	1.1	0
Pool #2 Phenolized Vaccine	1:10	0/12	—	—	7/12	6.0	9.7	★ ★	7
	1:1000	9/12	4.8	15.6	10/12	5.8	14.4	0.9	1
Pool #V Phenolized Vaccine	1:10	0/12	—	—	As for pool 2				7
	1:1000	9/12	6.2	12.0			14.4	1.2	1
Rabbit #V ₁ Phenolized Vaccine	1:10	0/12	—	—	7/12	6.0	9.8	—	7
	1:1000	4/12	6.2	5.4	5/12	7.8	5.3	1.0	1
Rabbit #V ₆ Phenolized Vaccine	1:10	0/12	—	—	See V ₃	See V ₃	See V ₃		7
	1:1000	0/12	—	—	See V ₃	See V ₃	See V ₃		5
Rabbit #IV Recovered from Jap Infection	1:10	1/12	—	—	8/12	—	—	10.5	7
	1:1000	3/11	—	—	8/12	—	—	2.1	5
Rabbit #V Recovered from Jap Infection	1:10	0/12	—	—	6/12	—	—	—	6
	1:1000	0/12	—	—	8/12	—	—	—	8

★ All mice received 0.5 ml of serum intravenously and were challenged at once with 10 and 100 LD₅₀ of the Ince strain (LD₅₀ 10⁻⁷). The number of 11 or 12 represents the total of mice injected at the above dilutions.

★ ★ Not computable

+ This rabbit was bled after 3 and 5 doses of phenolized vaccines instead of the 1 or 2 doses given to rabbits in Pools 1, 2.

Table 3

The protective effect of serum from rabbits after recovery from a living dose of the variant strains of Jap

Immune			Normal		
Immune Serum	Dilution Tested	Dead Tested	Dead tested	MPR	Σ
Jap A	Undiluted	1/10	7/12	3.9	6
Smooth	1:10	0/12	10/12	★	10
LD ₅₀ 10 ^{-0.7}	1:100	1/12	10/12	16.1	9
Immunogenic for Mice	1:1000	5/12	11/12	3.5	6
Jap B	Undiluted	2/12	7/12	5.5	5
Non-Smooth	1:10	1/11	10/12	5.4	8
LD ₅₀ 10 ^{-1.1}	1:100	0/12	10/12	★	8
Non-immunogenic	1:1000	1/12	11/12	14.4	10
Jap C	Undiluted	0/12	7/12	★	7
Smooth	1:10	0/12	10/12	★	10
LD ₅₀ 10 ^{-0.6}	1:100	2/12	10/12	★	8
Less immunogenic than A	1:1000	0/12	11/12	★	11
Jap H	Undiluted	0/12	7/12	★	7
Smooth	1:10	0/12	10/12	★	10
LD ₅₀ 10 ^{-4.6}	1:100	1/12	10/12	14.0	9
Immunogenic	1:1000	0/12	11/12	★	11

† All rabbits received sublethal doses of the living variants and were bled 10 to 14 days after infection.

★ Not computable

Table 4

The protective effect of serum from rabbits after recovery from two living doses of the variant strains of Jap

Immune			Normal		
Immune Serum	Dilution Tested	<u>Dead</u> tested	<u>Dead</u> tested	MPR	Σ
Jap A	Undiluted	0/12	11/11	★	11
	1:10	0/12	10/12	★	10
	1:100	2/12	9/12	6.0	7
	1:1000	1/12	11/12	9.0	10
Jap B	Undiluted	Toxic	11/11	★	★
	1:10	0/12	10/12	★	10
	1:100	0/12	9/12	★	9
	1:1000	1/12	11/12	27.	10
Jap C	Undiluted	0/12	11/11	★	11
	1:10	0/12	10/12	★	10
	1:100	0/12	9/12	★	9
	1:1000	1/12	11/12	21.9	10
Jap H	Undiluted	Toxic	11/11	★	★
	1:10	0/12	10/12	★	10
	1:100	0/12	9/12	★	9
	1:1000	2/12	11/12	7.0	9

★ Not computable

A. Protection Tests - Whole and Fractionated Serum.

This experiment was done in collaboration with a graduate student working with Dr. Mills. He is fractionating serum from infected rabbits using the methods developed by Cohn⁽⁸⁾. Fractions II and III represent gamma and beta globulins, Fraction IV,V and VI represent alpha globulin, albumin and non-protein constituents. In this preliminary test no attempt was made to refine the gamma or beta globulins. The protection tests show the protective effect of the whole serum compared to the effect of Fractions II,III and IV,V,VI.

The method for producing the serum was to infect the rabbits with 400 million of the respective variants of Jap and to bleed them after 10 days. A portion of the serum was kept frozen until the remaining serum was fractionated and ready to be tested. It may be seen from Table 5 that Jap H, A and B all gave Sigma values above 5 at dilutions of 1:10 but only Jap H gave a Sigma value above 5 at 1:1000.

Jap A II and III and Jap B II and III gave Sigma values of 6 and 1 respectively at 1:1000. Jap A IV,V and VI and Jap B IV,V and VI gave Sigma values of 0 and 4 respectively at 1:1000. The values given by Jap H II and III and IV,V and VI are discrepant since it would be expected that II and III were the protective part of the serum. Jap H II and III gave a Sigma value of 0 at 1:1000 and IV,V and VI gave a Sigma value of 5 at 1:1000. Jap B IV,V and VI also gave greater protection at 1:1000 than Jap B II and III. It seems possible that these results indicate incomplete separation of the serum fractions. This experiment is being repeated and will be reported later. In all cases the agglutination titers were negative in fractions IV,V and VI. The protective titer of Jap H and Jap A whole serum at 1:1000

-26-

was better than Jap B and this is in line with greater immunogenicity of H and A when given to mice in sublethal doses.

Table 5

Summary of the protective effect for white mice of whole serum and of fractions from recovered rabbits

Immune			Normal	
Source and Agg. titer	Dilution Tested	Dead Tested	Dead Tested	Σ
★ Jap H - S Serum 1:10, 240	1:10	0/12	8/12	8
	1:1000	3/12	9/12	6
Jap H II, III 1:5120	1:10	6/12		2
	1:1000	9/12		0
Jap H IV, V, VI 0	1:10	0/12		8
	1:1000	4/12		5
Jap A - S Serum 1:1,280	1:10	1/12		7
	1:1000	6/12		3
Jap A II, III 1:2560	1:10	0/12		8
	1:1000	3/12		6
Jap A IV, V, VI 0	1:10	8/12		1
	1:1000	9/12		0
Jap B - NS Serum 1:320	1:10	1/12		7
	1:1000	8/12		1
Jap B II, III 1:320	1:10	4/12		4
	1:1000	8/12		1
Jap B IV, V, VI 0	1:10	4/12		4
	1:1000	5/12		4

★ The immune serum was obtained from rabbits which had recovered from an inoculation of 400 million organisms of the strain designated. The letter S indicates that this was a smooth variant, NS, a non-smooth variant.

II. IMMUNITY STUDIES

B. Bactericidal tests:

The tests as outlined under Material and Methods have been continued using individual rabbit and human blood. Tables 6 and 7, give the figures obtained where normal, vaccinated and recovered human blood is inoculated with a virulent strain of Bacterium tularense and sampled after 24, 48 and 72 hours of incubation. Each blood sample was tested at two levels of inoculum, namely 400 organisms per ml and 4000 organisms per ml. The results are expressed in log numbers since analysis of the exact counts indicated that differences within logs were not significant. It may be seen from the tables that the bactericidal effect of human blood from vaccinated and recovered persons was very slight and there was slight difference between vaccinated and recovered. Probably most of the effect was due to the presence of agglutination in the vaccinated and recovered bloods. That there was a slight bacteriostatic effect is evident from Figure 1. The results on vaccinated and recovered rabbit blood are given in Tables 8 and 9 and Figure 2. In the case of the rabbit blood there was some reason to believe that the bacteriostatic effect was more marked in the recovered rabbits than in the vaccinated rabbits. Both the vaccinated and recovered blood contained agglutinins but the number of organisms in the recovered blood was consistently less at 48 and 72 hours than in the vaccinated blood. In no case however was the blood sterilized. Clearance studies, reported on page 43 this report indicate that in the mouse, removal of organisms from the blood stream is most effectively performed by the liver.

Table 6
Bactericidal effect of human blood

Blood tested	Agg. Titer	Hour of testing			
		0	24	48	72
Normal					
1	0	$2 \times 10^{2.5}$	3×10^2	1×10^4	2×10^6
2	0	2×10^2	4×10^2	1×10^4	5×10^5
3	0	2×10^2	2×10^2	2×10^5	3×10^3
4	0	2×10^2	2×10^2	1×10^2	3×10^2
5	0	2×10^2	2×10^2	2×10^2	2×10^3
8	0	3×10^2	3×10^2	1×10^3 $2 + 2 \log$	5×10^3 $3 + 1 \log$
Vaccinated				$2 + 1 \log$	$1 = \text{same}$
Time since 1				2 same	$1 = +3 \log$
vaccination					$1 = +4 \log$
1 (4 mo.)	1:320	3×10^2	9×10^2	2×10^3	3×10^2
2 (2 mo.)	1:160	3×10^2	5×10^2	4×10^3	5×10^3
3 (2 mo.)	1:160	2×10^2	2×10^2	1×10^3	3×10^4
4 (1 mo.)	1:640	2×10^2	2×10^2	1×10^2	1×10^2
5 (1/2 mo.)	1:640	3×10^2	2×10^2	5×10^2	3×10^3
6 (1/2 mo.)	1:160	3×10^2	3×10^2	6×10^2 $3 + 1 \log$	2×10^3 $2 = \text{same}$
Recovered				3 same	$3 = +1 \log$
Time since					$1 = +2 \log$
Recovery					
1 7 years	1:80	3×10^2	2×10^2	4×10^2	2×10^3
2 4 years	1:320	3×10^2	2×10^2	6×10^2	6×10^2
3 (1 1/2 mo.)	1:320	2×10^2	1×10^2	3×10^2	8×10^3
4 (9 mo.)	1:1280	2×10^2	2×10^2	9×10^1	1×10^2
5 (2 mo.)	1:2560	3×10^2	1×10^2	1×10^2	9×10^2
6 (2 mo.)	1:5120	3×10^2	5×10^2	8×10^2	5×10^2
7 (1 mo.)	1:640	3×10^2	4×10^2	2×10^2 0 same	4×10^2 $5 = \text{same}$
				$1 - 1 \log$	$2 = +1 \log$

* The heparinized blood was inoculated with a dilution of $5m$ to give a calculated dosage of 400 organisms per ml.

Table 7
Bactericidal effect of human blood

Blood tested	Agg. Titer	Hour of Testing			
		0	24	48	72
Normal					
1	0	2×10^3 *	3×10^3	1×10^5	7×10^6
2	0	2×10^3	1×10^3	3×10^4	4×10^6
3	0	2×10^3	2×10^3	9×10^3	3×10^4
4	0	2×10^3	1×10^3	2×10^3	1×10^4
5	0	2×10^3	2×10^3	1×10^4	6×10^4
6	0	6×10^3	1×10^4	1×10^4 $1 + 2 \log$	2×10^4 $4 + 1 \log$
Vaccinated				$3 + 1 \log$	$2 + 2 \log$
1 (4 mo.)	1:320	2×10^3	4×10^3	5×10^3 2 same	5×10^3
2 (2 mo.)	1:160	2×10^3	2×10^3	1×10^4	4×10^4
3 (2 mo.)	1:160	1×10^3	1×10^3	2×10^3	3×10^4
4 (1 mo.)	1:640	3×10^3	1×10^2	4×10^3	2×10^3
5 (1/2 mo.)	1:640	2×10^3	1×10^3	9×10^3	2×10^4
6 (1/2 mo.)	1:160	2×10^3	2×10^3	3×10^3 $1 + 1 \log$	1×10^4 2 same
Recovered				5 same	$4 + 1 \log$
1 7 years	1:80	3×10^3	1×10^3	2×10^3	1×10^4
2 4 years	1:320	2×10^3	9×10^2	2×10^3	5×10^3
3 1 1/2 years	1:320	1×10^3	1×10^3	1×10^2	6×10^4
4 (9 mo.)	1:1280	2×10^3	1×10^3	2×10^3	1×10^3
5 (2 mo.)	1:2560	2×10^3	1×10^3	5×10^2	9×10^3
6 (2 mo.)	1:5120	2×10^3	2×10^2	4×10^2	4×10^3
7 (1 mo.)	1:640	2×10^3	2×10^2	7×10^2 3 same	1×10^4 4 same
				$4 - 1 \log$	$3 + 1 \log$

* The heparinized human blood was inoculated with a dilution of Sm to give a calculated dosage of 4,000 organisms per ml.

Table 8

Bactericidal effect of rabbit blood

Rabbits Category	Treatment	Agg. Titer	Hour of testing			
			0	24	48	72
Normal						
1	None	0	4×10^2 *	8×10^2	1×10^3	8×10^3
2	None	0	3×10^2	5×10^2	$\frac{1 \times 10^4}{+1}$	$\frac{4 \times 10^4}{+1}$
Vaccinated					$\frac{+2}{+2}$	$\frac{+2}{+2}$
1	Jap A†	1:320	2×10^2	2×10^2	1×10^2	4×10^2
2	Jap B	1:160	3×10^2	3×10^2	1×10^2	1×10^2
3	Jap H	1:80	3×10^2	5×10^2	$\frac{8 \times 10^2}{3 \text{ same}}$	$\frac{5 \times 10^2}{2 \text{ same}}$
Recovered						$\frac{1}{1} + 1$
1	Jap A††	1:1280	4×10^2	1×10^2	4×10^1	2×10^1
2	Jap B	1:320	4×10^2	2×10^2	8×10^1	3×10^1
3	Jap H	1:10,240	3×10^2	1×10^2	$\frac{4 \times 10^1}{3 - 1}$	$\frac{5 \times 10^1}{3 - 1}$

* The heparinized blood was inoculated with a suspension of Sm to give a calculated dosage of 400 organisms per ml.

† Rabbits were injected with 1 ml of phenolized vaccine of the strain designated containing 400 million organisms per ml.

†† Rabbits which had recovered after an infecting dose of 400 million living organisms of the strain designated.

Table 9

Bactericidal effect of rabbit blood

Rabbits Category	Treatment	Agg. Titer	Hour of Testing			
			0	24	48	72
Normal						
1	None	0	$3 \times 10^{3.2}$	3×10^3	4×10^3	1×10^5
2	None	0	3×10^3	2×10^3	3×10^4 $\frac{1+1 \log}{1 \text{ same}}$	5×10^4 $\frac{1+2 \log}{1+1 \log}$
Vaccinated						
1	Jap A [†]	1:320	2×10^3	5×10^2	4×10^2	4×10^3
2	Jap B	1:160	2×10^3	1×10^3	3×10^3	4×10^3
3	Jap H	1:80	2×10^3	2×10^3	3×10^3 $\frac{1-1 \log}{2 \text{ same}}$	4×10^3 $\frac{3 \text{ same}}$
Recovered						
1	Jap A ^{††}	1:1280	4×10^3	8×10^2	4×10^2	2×10^3
2	Jap B	1:320	3×10^3	1×10^3	8×10^2	3×10^3
3	Jap H	1:10,240	3×10^3	7×10^2	2×10^2 $\frac{3-1 \log}{2-1 \log}$	2×10^1 $\frac{2-1 \log}{1-2 \log}$

* The heparinized blood was inoculated with a suspension of Sm to give a calculated dosage of 4,000 organisms per ml.

+ Rabbits were injected with 1 ml of phenolized vaccine of the strain designated containing 400 million organisms per ml.

†† Rabbits which had recovered after an infecting dose of 400 million living organisms of the strain designated.

Figure 1 The effect of normal, vaccinated and recovered human whole blood when inoculated with 400 organisms per ml and sampled at the time shown.

Figure 1 HUMAN BLOOD

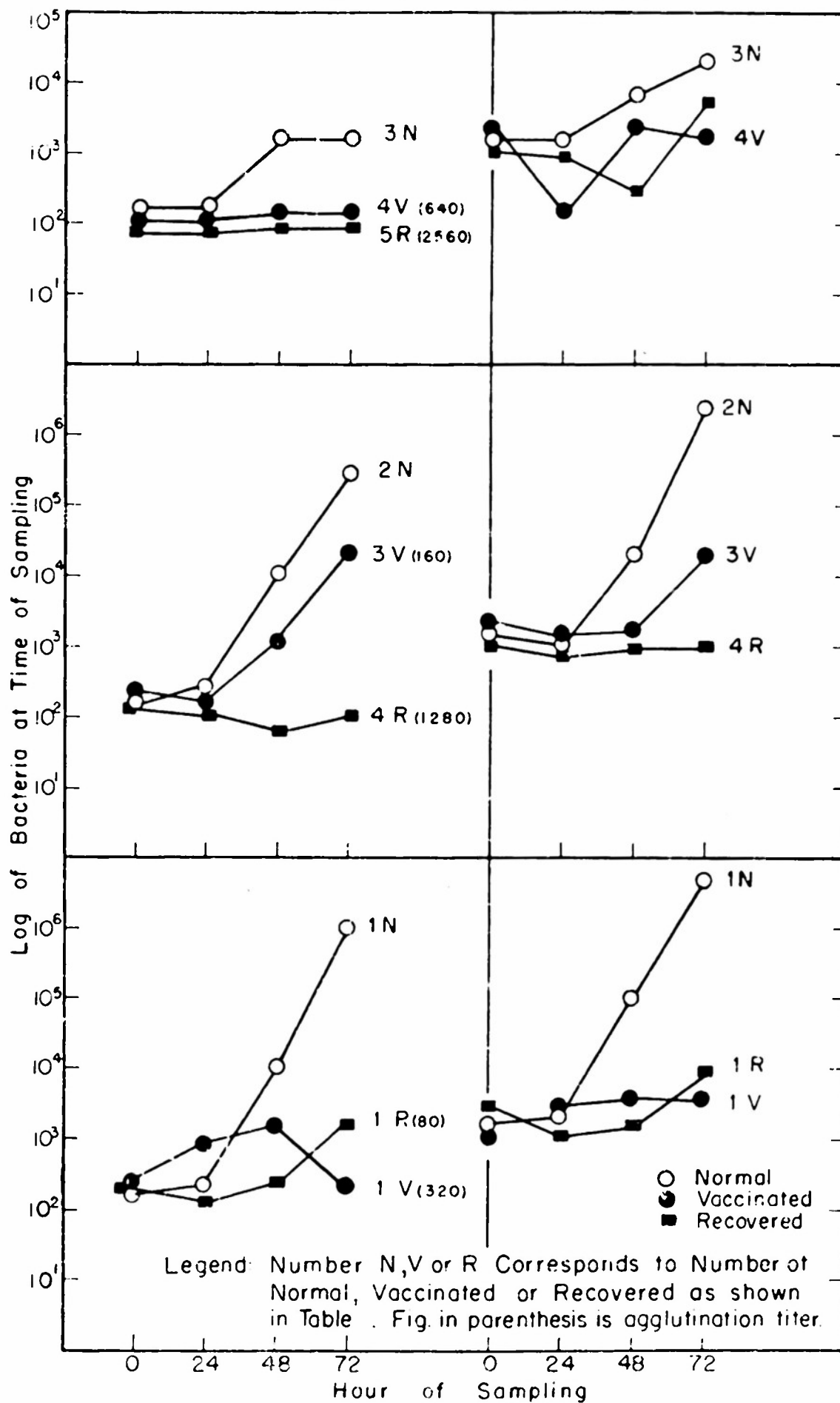
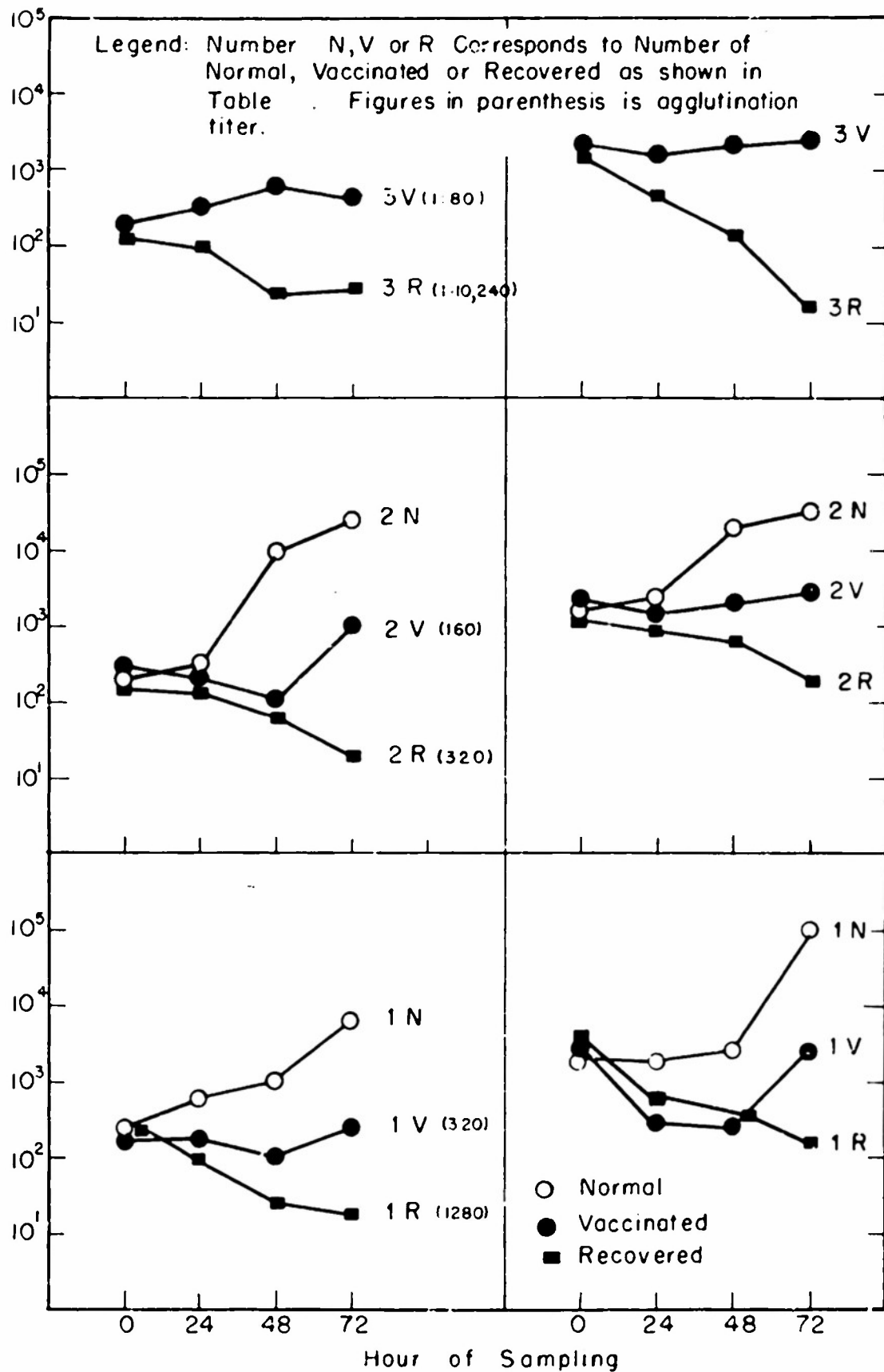


Figure 2 The effect of normal, vaccinated and recovered rabbit whole blood when inoculated with 400 organisms per ml and sampled at the time shown.

Figure 2 RABBIT BLOOD



II. IMMUNITY STUDIES

C. Ascoli tests:

Recent reports by Larson⁽⁵⁾, ⁽⁹⁾ have indicated that this test could be used to identify an antigen present in animals dying of tularemia or plague. The antigen from tularemic animals and from cultures is thermostable, specific, extractable with ether and is acetone insoluble. It is stable enough to remain reactive in dead animals for 14 days. The chemical nature of this antigen was not defined nor was the time of its appearance or the relation of the antigen to the presence of antibodies in the animal elucidated.

It seemed worth while in connection with other studies on the virulence and immunogenicity of various strains of Bacterium tularense to ask the following questions: 1. When does the Ascoli antigen appear in the infected animal, in the challenged immune animal, and what is its relation to antibody occurrence; 2. What is the relation to virulence of the strain used to infect the mouse; 3. Is the Ascoli antigen immunogenic for white mice?

In preliminary experiments mice injected for other experiments were routinely examined after autopsy for the presence of the thermostable antigen. The liver and spleen were pooled, ground and mixed with four ml of saline thus giving roughly a 1:5 dilution of tissue. This was heated in flowing steam for 30 minutes, centrifuged and the supernatant used for precipitin tests. The sera used are normal rabbit serum and immune rabbit serum with an agglutinin titer of 1:2560. A summary of this experiment is shown in Table 10. The results shown in Table 10 suggested that strains of both high and low virulence when

Table 10

The number of mice showing the Ascoli antigen
following death from challenge

Challenge organism and LD ₅₀	Day of death	<u>Positive Tested</u>	Precipitin Titer
Jap H 10 ^{-4.5}	1-3	2/14	1:16
Jap H	4-6	6/7	1:16 to 1:64
Jap 1 10 ^{-2.4}	1-3	4/9	1:2
SmA 10 ^{-9.5}	1-3	6/6	1:2 to 1:64
SmA	4-8	21/27	1:2 to 1:64
Ince 10 ⁻⁷	4-8	7/7	1:16 to 1:64
Ince + normal rabbit serum	4-8	13/16	1:8 to 1:64
Ince + immune rabbit serum	4-8	1/7	1:64

given in sufficient amounts to kill mice produced the Ascoli antigen in the tissues. It may be significant that only one out of seven mice which had received immune serum contained the Ascoli antigen.

In attempting to answer the question of when the antigen appears and whether its appearance bears any relation to virulence and agglutinating antibodies a series of mice were inoculated with strains of low, moderate, and high virulence and were sacrificed daily in groups of three. They were bled from the heart for serum for agglutination tests and after washing the liver and spleen to remove adherent organisms the tissues were pooled and treated as given above. The supernatant was used for the precipitin test and the mouse serum was used for agglutination tests in the usual manner. A summary of this experiment is given in Table 11. The figures are pooled for each 48 hours and the titers represent the reciprocals of the highest and lowest recorded in the various groups. The results suggest that the antigen occurs in mice given lethal doses of the virulent organisms, Sm 1 and BB, LD₅₀ 10^{-9.3}, when the animals are tested on the third or fourth day. The animals given strains of lower virulence, Jap 1, Jap 2, and SmO, do not show the antigen unless given lethal doses. Jap H on the other hand which is of very moderate virulence gave some evidence of the antigen in sublethal doses. Since this organism is known to multiply in the mouse, the multiplication of the organism may be necessary for the production of the antigen. The mice challenged with the BB strain after immunization showed only two out of fourteen positive.

All of the mice developed agglutinins and only in the case of the Jap H mice were the agglutinins low when the antigen was present and

Table 11

Development of the Ascoli antigen in mice

Strain—Dose Dilution infected	24-48		72-96		5th-6th day		7th-8th day		9th-10th day		11th-12th day	
	P/T	Aggr	P/T	Aggr	P/T	Aggr	P/T	Aggr	P/T	Aggr	P/T	Aggr
Jap 1 10 ^{-2.4}												
	1/7 (---)	---	8/8 (4-16)	---	0/1 (---)	---						
	0/6 (---)	---	0/6 (---)	---	0/6 (---)	16	0/6 (---)	16-64	0/6 (---)	64	0/3 (---)	64
Jap 2 10 ⁰												
10 ⁻¹	0/6 (---)	---	0/3 (---)	---	0/6 (---)	32-64	0/6 (---)	64	1/6 (8)	32	0/3 (---)	32
10 ⁻²	0/6 (---)	---	0/3 (---)	---	0/6 (---)	2-4	0/6 (---)	2-4	0/6 (---)	2-16	0/3 (---)	32
Sm 1 10 ^{-2.3}												
10 ⁻²	0/6 (---)	---	7/8 (2-32)	4-8	2/2 (4)	Not tested						
Sm 2 10 ^{-2.2}												
10 ⁻²	0/6 (---)	---	0/6 (---)	8-32	0/8 (---)	16-64	0/3 (---)	16				
10 ⁻⁴	0/6 (---)	---	0/6 (---)	4-16	0/6 (---)	16-64	0/6 (---)	8				

Table 11 (cont.)

Strain--LD ₅₀ Dilution injected	24-48		72-96		5th-6th day		7th-8th day		9th-10th day		11th-12th day	
	P/T	AGG	P/T	AGG	P/T	AGG	P/T	AGG	P/T	AGG	P/T	AGG
Jap H 10 ^{-4.6}												
10 ⁻³	0/3 (---)	---	4/8 (2-64)	0-8	9/12 (2-64)	8-32	2/3 (2)	32				
10 ⁻⁵	0/3 (---)	---	1/7 (8)	0-8	3/6 (0-16)	8-16	2/6 (0-8)	32-64	0/3 (---)	64	0/6 (---)	128
BB 10 ^{-9.3}												
10 ⁻⁹	0/3 (---)	---	10/11 (8-64)	16-32	1/1 (4)	---	0/4	32				
Immune Mice [*] BB												
10 ⁻⁴	0/4 (---)	32	2/10 (0-4)	64-128	0/4 (---)	128						

+ P/T () designates positive over tested and the number in parenthesis gives the precipitin titer of the antigen in the presence of high titered rabbit serum.

† The figure is the reciprocal of the agglutinin titer of the mouse serum.

* These mice had been immunized with Jap H and were challenged with the BB strain.

high when the antigens were absent.

Four strains of varying virulence were extracted by Larson's method. A standard suspension of each strain was used and the various extracts as shown in Table 12 were used in precipitin tests. Our results agreed with Larsons in that heat and ether extraction gave reactive antigens. Jap H and Sm, both of them immunogenic but Jap H of lower virulence, gave a higher average titer for all extracts. Jap C and SmO, which are non-immunogenic and of lower virulence gave low titers or none at all. Larson showed that the completely avirulent 38 gave less antigen than the virulent strains if extracted with ether but the same when extracted with heat alone. We found this was true for Jap C but not SmO. A reactive antigen was apparently present with ether extract alone from SmO but not in the other extracts. The most reactive antigen was obtained from cultures by means of ether extraction followed by acetone precipitation (D_1). These experiments are preliminary and should be pursued using smooth and non-smooth variants of varying degrees of immunogenicity and virulence.

In connection with other Ascoli tests a large amount of the antigen was prepared for infection into white mice to test the immunogenicity of the antigen for this animal. Larson ⁽⁴⁾ had reported that ether extracted or heat and ether extracted cultures provoked both agglutinins and precipitins in rabbits, a maximum titer of 1:1280 agglutination and 1:512 precipitation.

The antigen for these tests was prepared as before by the inoculation of 0.5 ml of living Jap H containing 400,000 cells into each mouse. The mice were killed 24, 48, 72, and 96 hours after injection

Table 12

Pricipitin tests and Ascoli antigen extracts
from cultures of variant strains

Means of Extraction	Strains used and LD ₅₀			
	Jap H 10 ^{-4.8} S	Jap C 10 ^{-0.8} NS	Sma 1C ^{-9.8} S	SrO 10 ^{-8.8}
A1 Culture, super- nate cantrifuged	0	0	8.	0
A2 Supernate after heat	32	32	64	0
B1 Supernate A optated acetone	0	0	0	0
B2 Heat plus acetone	8	32	16	0
C1 Supernate after ether	64	16	32	16
C2 Heat and ether	32	16	64	0
D1 Ether and acetone	256	16	128	0
D2 Ether plus heat plus acetone	64	16	128	0
Average titer	76	22	63	

and the tissues from each lot were ground and treated as follows:

- A. Heated one-half hour in flowing steam and centrifuged and the supernatant saved (Heat).
- A₂. As above but adjusted to pH 3.0, precipitated with cold methyl alcohol, pH adjusted to 7.0, and allowed to stand over night. The precipitate was discarded and the pH adjusted to 4.5. Equal parts of acetone added and the precipitate dissolved in one-half the original volume (HAc).
- B. The ground tissues were extracted with ether following Larsons directions and divided into two parts. One part was used without further treatment (E). The other part was heated and precipitated with acetone (HE).
- C. The ground tissues were extracted with ether as before and the aqueous phase was divided into two parts. One part was precipitated with cold methyl alcohol. The precipitate was dissolved in normal saline to one-half the original volume (AlE). Not all of this precipitate dissolved until it was treated for twenty four hours in the refrigerator with buffered saline pH 8.0 (AlEB). The second part of the aqueous phase from the original extract was precipitated with acetone and the precipitate dissolved in one-half the original volume in saline (AcE).

These various preparations were injected intraperitoneally in 0.5 ml amounts into 18 mice and they were given four injections on alternate days. Ten days after the final injection; the mice were challenged with the virulent strain Sm and an LD₅₀ determined using 6 mice per dilution.

The mice evidenced no immunity since the LD_{50} of the test mice varied from $10^{-8.7}$ to $10^{-9.0}$ and the control mice showed an LD_{50} of $10^{-8.8}$. There was no significant prolongation of life when the test mice were challenged with $100 LD_{50}$, there was one day and 2.6 days prolongation at $10 LD_{50}$ and $1 LD_{50}$ respectively. It is doubtful if any real significance can be attached to this prolongation of life.

The results are summarized in Table 13. Ascoli tests performed on all the extracts were negative. This may be explained by the fact that we have rarely recovered the Ascoli antigens from mice which were not dead or dying from an overwhelming infection.

C-7

-48-

Table 13

Summary of the results of the injection of tissue
extracts prepared by Larsons method

Extract	Dead [*] Tested	ADD †	LD ₅₀	Ascoli test
Normal	14/17	4.7	10 ^{-8.8}	Neg.
Heat ††	16/18	6.5	10 ^{-9.0+}	Neg.
(HAc)	15/18	5.9	10 ^{-9.0+}	Neg.
(E)	15/18	7.8	10 ^{-9.0+}	Neg.
(HE)	11/18	6.7	10 ^{-9.0+}	Neg.
(AIE)	15/18	5.8	10 ^{-9.0+}	Neg.
(AIEB)	14/18	5.8	10 ^{-8.7}	Neg.
(AcE)	16/18	6.1	10 ^{-9.0}	Neg.

* The total number injected with 100, 10 and 1 LD₅₀ after challenge strain is shown as dead/tested.

† The average day of death is the average day at which all mice died at the three levels tested.

†† The extracts were prepared and coded as given in the text.

II. IMMUNITY STUDIES

D. Mouse Clearance:

We have previously made quantitative studies on the progress of infection in normal and immune rats following inoculation of multiple lethal doses of virulent strains of Bacterium tularensis.⁽¹⁰⁾ The results demonstrated that immune animals were able to limit the multiplication of the organisms in tissue while in normal animals multiplication continued unchecked until death. Woodward,⁽¹¹⁾ in our laboratory, showed that mice infected with virulent strains died with an overwhelming infection on the fourth to sixth day, whereas mice which had recovered from infection with strains of low virulence were able to limit the multiplication of virulent challenge strains on the fourth to fifth day. The organisms invaded the tissues, multiplied, then decreased in numbers, remaining only in the spleen for several weeks. The sharp limitation of numbers on the fourth to sixth day was striking evidence of the ability of the organisms to overcome infection.

Many workers have shown that after intravenous inoculation of normal animals, there is an initial decrease of the organisms in the blood, then an increasing bacteremia until death. In view of the differences shown between the normal and immune animals in previous experiments, it seemed worth while to study the distribution of Bacterium tularensis in normal and immune mice, following intravenous inoculation of large numbers of organisms.

In the experiments we are now reporting, large numbers of virulent organisms were inoculated into the tail vein of normal or immune mice.

The immune animals had recovered from a sublethal infection. Approximately two to four million organisms were inoculated into the mice. In each experiment, two mice were killed at once, at 4, 6, and 24 hours after inoculation. Blood was obtained from the heart for culture, and the liver, spleen, and lungs were weighed, ground and cultured. All plate counts were made by surface streaking on GCBA plates. The number of organisms per gram, and the number per tissue was recorded. The latter figure is obtained by multiplying the number per gram by the weight of the organ. The total number of organisms recovered was the sum of those obtained from blood, liver, spleen, and lungs, and did not therefore represent all of the organisms present in the animal.

Figure 3 shows the results obtained in the earlier work referred to, and shows the behavior of virulent organisms inoculated into normal mice, and into mice which had received an immunising dose of the immunogenic strains of lowered virulence. It is apparent that the organisms multiplied during the five days of sampling, but to a lesser degree in the immune than in the normal mice. The differences in survival should be noted. All normal mice were dead on the fourth day whereas the immune mice gave from 92 to 100 per cent survival.

In the present work we attempted to find out what differences were apparent between the normal and immune animals in the period immediately after introduction of the organisms in the blood stream.

Figure 4 shows the per cent of the total organisms recovered from the blood, liver, and spleen in the respective tissues of mice and the per cent of the total in the lung, liver, and spleen.

**Figure 3. The multiplication of a virulent
challenge strain in normal and immunized
mice.**

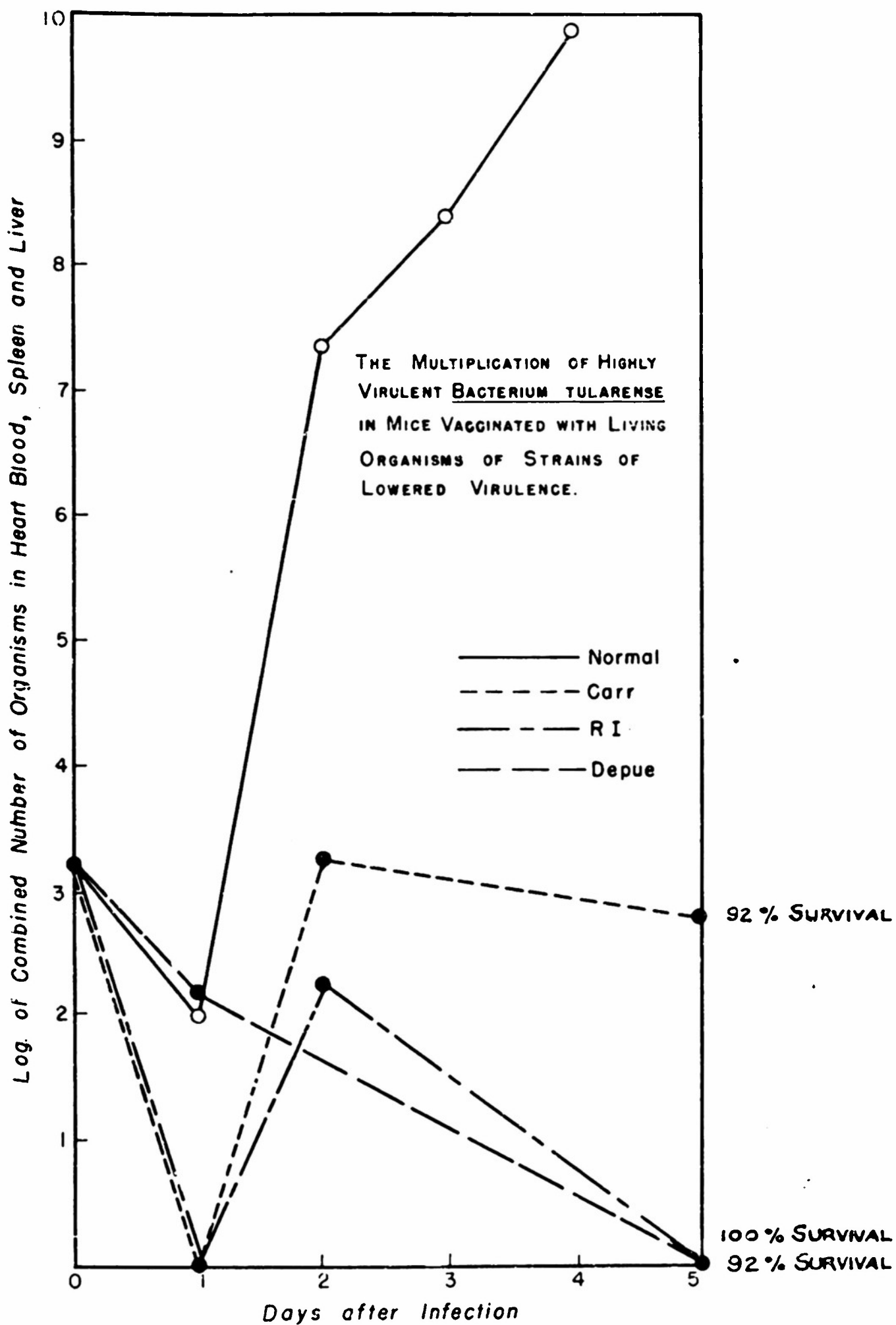
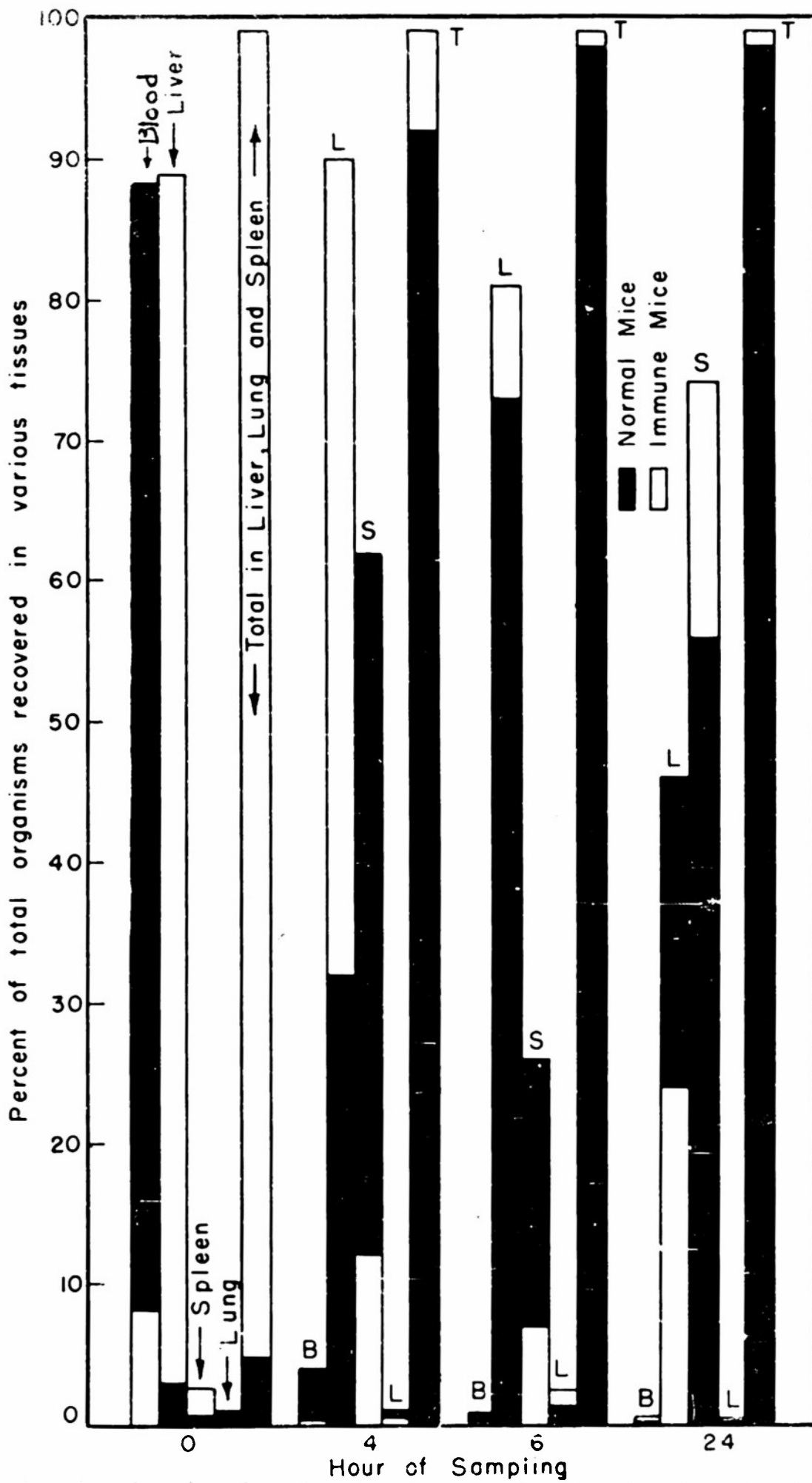


Figure 4: The per cent of the total number of organisms recovered from the respective tissues and the per cent of the total in the liver, lung, and spleen - of normal and actively immunized mice.



At the first period within five to fifteen minutes after injection, 89 per cent of the organisms could be recovered from the blood in the normal mice. In the immune mice there seemed to be a striking and immediate concentration of organisms in the liver. At the fourth hour, the organisms had very largely been concentrated in the spleen in the normal mice, although the per cent of organisms recovered had increased in the liver also. The liver of the immune mice still retained most of the organisms recovered. After the sixth hour the per cent of organisms has increased in the liver, and decreased in the spleen, whereas at 24 hours, the reverse is true. The per cent of organisms has increased also in the spleen in the immune animals.

The total number recovered as determined by figuring the per cent recovered from the total inoculum was less in the immune than in the normal. As, for instance, 60 and 70 at the 0 hour for the normal, and 26 to 28 for the immune. The percentage recovered becomes progressively less for the normal and immune to the sixth hour when multiplication starts. The details of five experiments representing the average figures on ten normal and ten immune mice sacrificed at the indicated hours are given in Table 14. It may be seen that the total number of organisms cultured from the immune mice at 24 hours is materially less than the number from the normal mice.

The immune mice had agglutinins in the serum and it is reasonable to suppose that the organisms would be agglutinated in the slow flowing blood of the liver sinuses and spleen and would be readily phagocytosed. This probably accounts for the rapid removal of the

Table 14

Clearance tests in mice
Average of five typical experiments in actively immunised mice

Category and Hour	Blood		Liver		Spleen		Lung		Total Recovered	Per cent in lung, liver and spleen
	Per tissue	% recovery	Per tissue	% recovery	Per tissue	% recovery	Per tissue	% recovery		
0										
Normal	144	89.	11.8	2.7	2.82	1.7	2.5	1.5	161	4.3
Immune	8.4	8	94.0	89.0	3.0	2.8	0.7	0.6	105	99.0
4										
Normal	5.13	3.9	43.0	32.4	82	62	1.1	0.8	132	92
Immune	0.01	0.01	95.0	91.4	14	12.8	0.53	0.4	109	99
6										
Normal	0.72	0.08	597	73.0	211	26	9.9	1.2	821	99
Immune	0.012	0.004	256	81.0	20.8	7.2	7.7	2.6	289.6	98
24										
Normal	101.	0.6	6580	41	8980	56	88.6	0.5	15,812	98
Immune	0.1	.001	1154	24	3550	74	22	0.4	4720	99

The figures represent the average number of organisms isolated per tissue $\times 10^4$. The total number of mice sacrificed at each hour was 10 so that each count on immune or normal mice represent the average of organisms found in 10 mice in the respective tissues.

organisms from the blood of the immune animal, but it is more difficult to account for the suppression of multiplication in the immune animal at the 6 and 24 hour intervals, since our studies have shown only slight bacteriostatic effect of whole blood.

Our results on normal mice are very similar to those of Martin, et al.,⁽¹²⁾ who showed that high proportion of both pneumococci and staphylococci are concentrated in the splanchnic area of rabbits during fatal septicemias. Our immune animals behaved toward virulent organisms much as the normal rabbits behaved toward the relatively avirulent staphylococci. Our results and theirs point to an initial concentration of organisms in the liver and spleen in normal animals followed by multiplication in the same areas with an increasing septicemia from washing out of the organisms into the blood stream. In immune animals the same procedure appears to occur after the initial more rapid clearance of the blood except that the degree of multiplication is less, and there is a gradual, but continued, decrease in multiplication of organisms until the animals recover.

Subsequent clearance experiments carried out in mice passively protected by immune serum from rabbits which had recovered from a sublethal infection with the strain Jap, revealed that the organisms were concentrated in the liver as before in the immune mice within 10 to 15 minutes after injection. After the sixth hour, multiplication took place in the liver and spleen and in these two series the multiplication in the immune mice after 24 hours was less than in the normal but the difference was much less striking than in the actively immune mice. These results are graphically shown in Figure 5.

Figure 5: The per cent of the total number of organisms recovered from the respective tissues of normal and passively immunized mice.

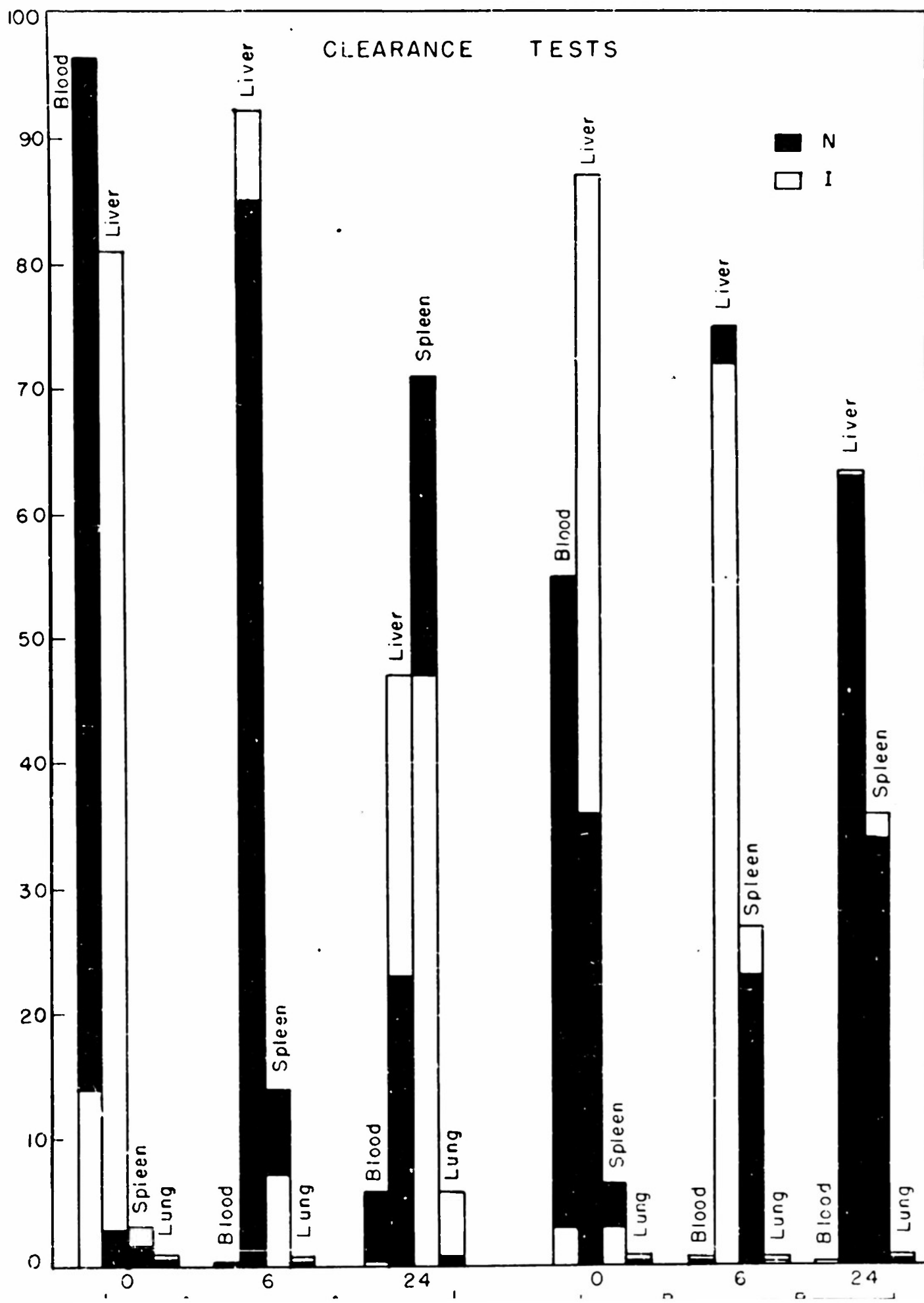
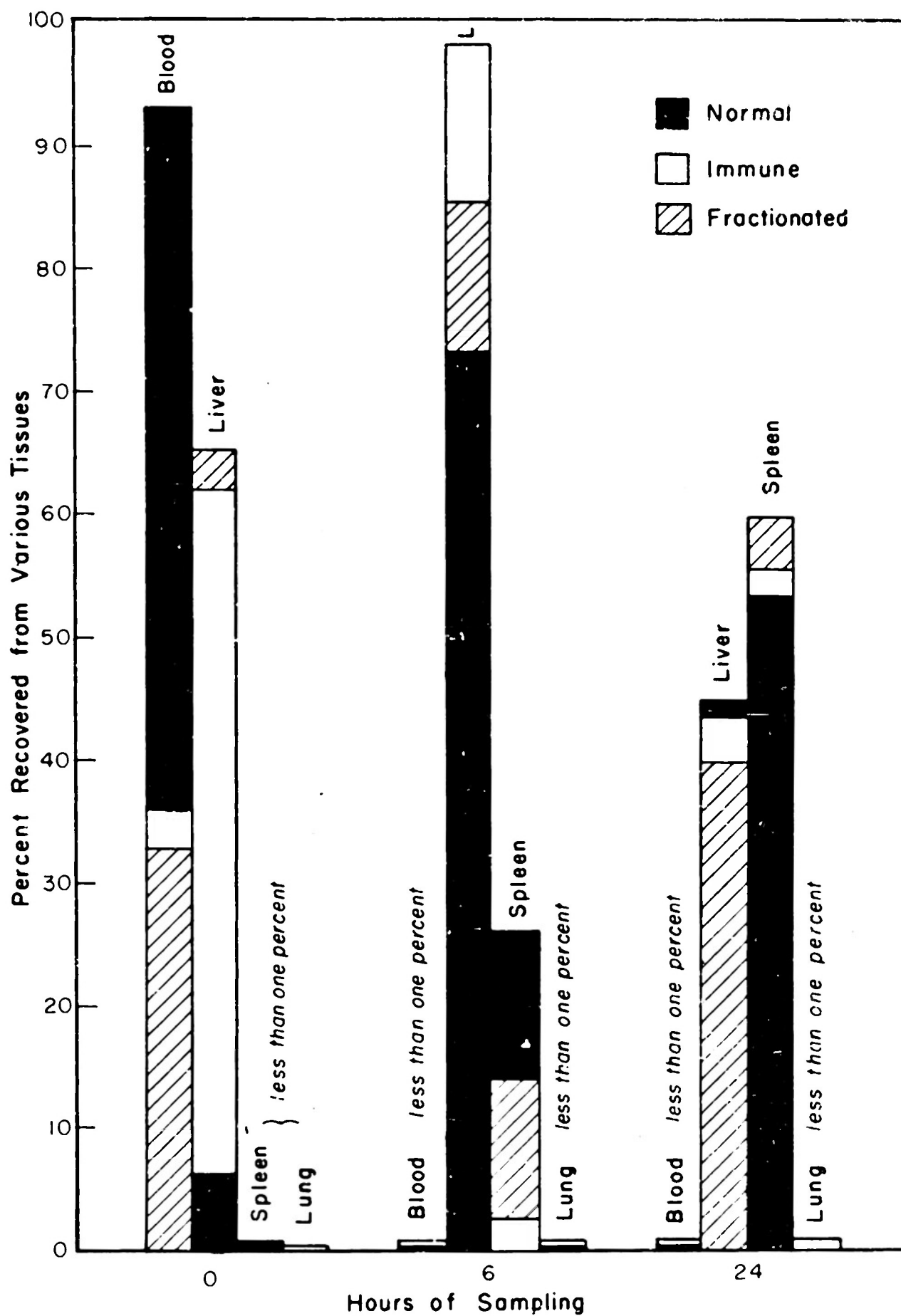


Table III and IV in the appendix show the detailed counts and percentages.

Passive clearance was also done on immune serum which had been fractionated. The fraction used gave the same general results as the whole immune serum as seen from Figure 6. The details of this experiment are set forth in Tables V and VI in the appendix.

Figure 6: The per cent of the total number of organisms recovered from the respective tissues after the injection of normal, immune, and fractionated immune serum into mice.



III. VARIATION STUDIES

Studies on variation of certain strains of Bacterium tularensis are being continued with the hope of correlating the relation of colony type, immunogenicity, antigenicity and virulence.

The work reported here is a continuation of the studies on variation given in the report for the first six months of the project. They will be discussed under the following topics: A. The antigenic response to variant strains in rabbits and rats, B. The behavior of smooth virulent forms in white mice and rats, C. The multiplication of variant strains in white mice. The details of these experiments are given in the appendix, the methods and results will be summarized here.

A. The antigenic response to variant strains in rabbits and rats.

We have previously reported the selection of smooth and non-smooth colonies from a variety of parent strains of Bacterium tularensis using the methods described by Werner Braun in numerous publications. These variant strains have been shown to be divisible into two groups on the basis of their properties in white mice.

<u>Group I</u>	<u>Group II</u>
1. Colony type smooth	1. Non-smooth
2. Virulent for white mice to some degree	2. Avirulent
3. Immunogenic for white mice	3. Non-immunogenic
4. Toxic for white mice	4. Non-toxic
5. Multiply in mice after small inocula.	5. Multiply poorly.

The properties of colony type and immunogenicity in many different species of microorganisms are linked with the presence or absence of certain antigens. These studies on Bacterium tularensis are attempts to

find evidence of such antigen association. Studies by Wright,⁽¹³⁾ Nicholes⁽¹⁴⁾ and ourselves have not indicated that the tularensis carbohydrate is lacking in avirulent cells. Larson also has demonstrated that the heat stable antigen of the Ascoli test is present in cultures of avirulent cells. That the avirulent cells behave differently from the virulent cells can be readily seen from the following experiment.

The antigenicity and immunogenicity of the variants chosen were tested on the susceptible rabbit and the more resistant rat in the following experiment. The antigenicity of the strains was measured by the agglutinin titers after infection and the immunogenicity by the resistance of the animals to challenge with a virulent strain.

The variants chosen were Ri 1 and Jap H representing members of Group I, smooth, partially virulent, and immunogenic for white mice. Ri 2 and Jap B were members of Group II, non-smooth, avirulent, non-immunogenic.

Five animals were injected with each variant. They received 3 intravenous injections on alternate days, containing 2 to 4 million organisms. Agglutination titers were determined after each injection series at 2, 4, 6, and 10 days. At 21 days the injection series was repeated and the agglutination titers were determined after 27, 29, 31 and 35 days after the first injection or 2, 4, 6, and 10 days after the second injection. All of the animals were bled from the heart on the 35th day to obtain serum for dissociation experiments.

On the 38th day all of the animals together with normal controls were challenged with a virulent strain, Sm A. The rats received

0.5 ml containing 400 million cells and the rabbits received 40 cells administered intraperitoneally. The individual titers are given in the appendix, Table VII and Table VIII. Table 15 and figure 7 and figure 8 give a summary of the results. The average titer for the 5 animals in each case was obtained by averaging the number of the tube in the series corresponding to the respective titer. The actual average titer was then determined. Ri 1 produced the highest titer in rabbits and Jap H the highest in rats following the first series of injections. Ri 2 and Jap B, the non-smooth variants, induced only a meager response in rabbits and a somewhat higher response in rats. In neither case was the titer as high as in the Ri 1 and Jap H animals.

The second series of inoculations evoked a rise of titer in rats with all variants, but in the case of Jap H the peak did not rise above the level attained on the 10th day after the first series. No marked rise was observed in rabbits after the second series of injections.

Although in rats Ri 1 and Jap H gave greater response than Ri 2 and Jap B, the contrast was not as great as in rabbits. This may be seen from figures 7 and 8.

The details of the results of challenge in the rats and rabbits are given in Table IX in the appendix. None of the rabbits survived 100 mouse LD₅₀ of the virulent strain though there was a slight prolongation of the average day of death in all of the rabbits. The rats survived 10 million mouse LD₅₀ with one exception. This animal died on the same day as the controls.

Table 15

Comparison of the Reciprocal of the Average
Agglutinin Titers in Rabbits and Rats

Vari- ant	Animal	Days after initial injection								
		2	4	6	10	21	27	29	31	35
S† Ri1 LD ₅₀ ★ 10 ^{-8.1}	Rabbit	0	48	1280	28672	3584	2304	3072	2560	1304
	Rat	24	128	2048	5120	1024	4068	6144	3072	2560
NS Ri2 10 ^{-0.8}	Rabbit	0	0	0	20	20	5	4	0	0
	Rat	56	64	112	128	160	320	320	320	112
NS Jap B 10 ^{-1.1}	Rabbit	0	20	24	28	12	16	24	0	0
	Rat	48	112	640	576	448	1292	1024	1792	1024
S Jap H 10 ^{-4.8}	Rabbit	0	24	570	448	160	224	160	144	192
	Rat	0	640	10240	12288	6144	3072	5120	3328	3328

† S - Smooth, NS - Non Smooth

★ The mouse LD₅₀ is given for the various strains

Figure 7: The antigenic response in
rabbits to Bacterium tularensis
variants

The Antigenic Response Induced in Rabbits by Four Variants

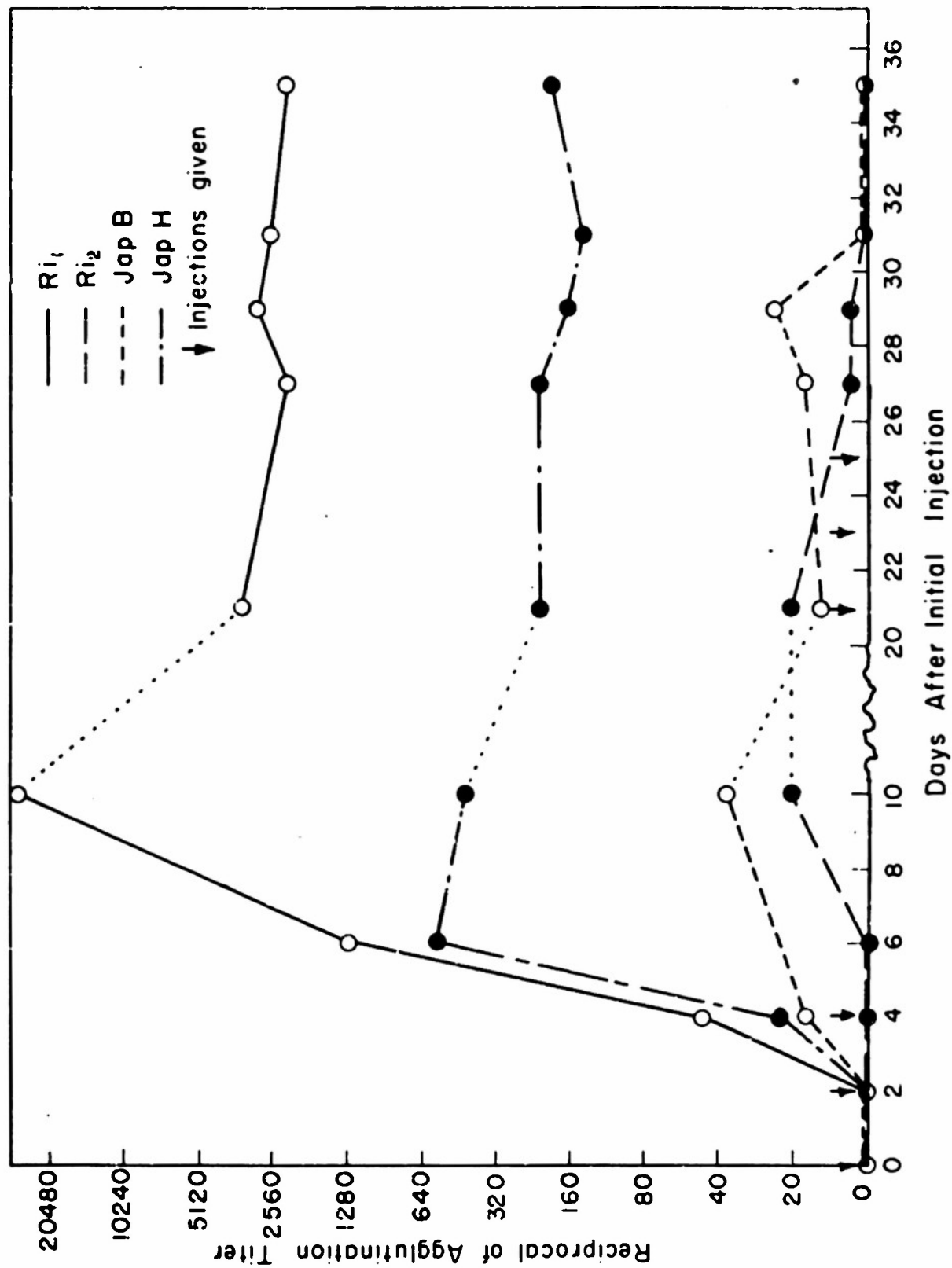


Figure 8: The antigenic response in rats
to Bacterium tularensis variants.

The Antigenic Response Induced in Rats by Four Variants

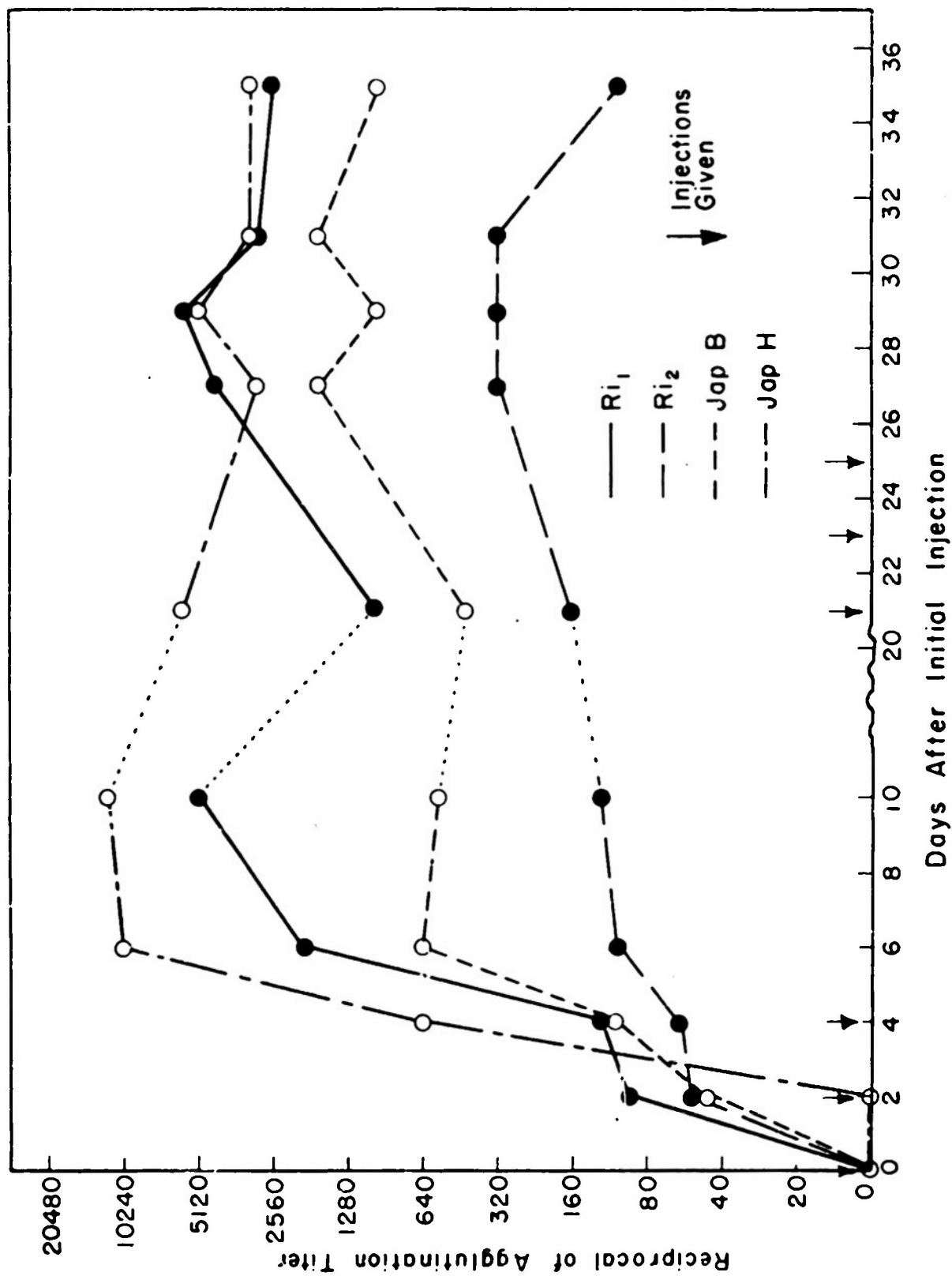


Table 16 gives a comparison of the average agglutinin titer and the average day of death in both groups of animals.

There seems to be evidence here that the smooth strains Ri 1 and Jap H have an agglutininogen which is more active or is lacking in Ri 2 and Jap B. The antigen which is responsible for immunogenicity in rats is apparently present in both smooth and non-smooth strains, whereas the antigen responsible for protection of mice is largely lacking in the non-smooth strains. Rabbits as usual can be shown to respond with agglutinins but are not immune to challenge doses.

Table 16

Comparison of Average Agglutinin Titer
with Average Day of Death following
Challenge with Sm A

Variant	Rabbits			Rats		
	Titer (1)	Dead Tested	ADD (2)	Titer (1)	Dead Tested	ADD (2)
R1 ₁	2304	5/5	6.8	3328	0/3	Survived
R2	0	4/4	5.3	160	1/3	2.0
Jap B	0	5/5	5.6	1024	0/5	Survived
Jap H	160	4/4	5.4	5120	0/2	Survived
Normal	0	5/5	3.6	0	4/4	2.0

(1) Determined 3 days prior to challenge. Represents average titer of animals actually challenged.

(2) Average day of death of animals challenged.

III. VARIATION STUDIES

B. The behavior of smooth virulent forms in white mice and rats.

The Sm 1 variant was selected for these experiments which were designed to determine if there was any dissociation to non-smooth forms in normal, highly susceptible white mice, in mice infected and then treated with streptomycin, in mice immunized by an infection which had been held in check by streptomycin.

Sm 1 is a variant selected for smoothness and virulence from the parent virulent Sm strain which is predominantly smooth but which dissociates when stored in broth into smooth, non-smooth and mucoid colonies.

Fifty nine mice were injected intraperitoneally with two to four hundred organisms. Forty nine mice received 10,000 units of dihydro-streptomycin intraperitoneally on the second and third days after infection, 2000 units on the fourth and seventh days. Two infected untreated and two treated mice were sacrificed at 0, 24, 48, 72, and 96 hours after infection. Impression smears of the heart blood and cultures from the emulsified liver and spleen were made on Snyders agar and on GCBA. The plates were incubated 72 hours at 37° C and examined after flooding with crystal violet as described in the report dated December 31, 1951.

In order to test the immunogenicity of Sm 1, two weeks after the injection of Sm 1, 29 of the surviving treated mice were challenged with 0.5 ml of Sm 1 (SS) diluted to 10^{-6} , 10^{-7} , 10^{-8} . As a control 30 normal mice were injected with Sm 1 in dilutions of 10^{-8} , 10^{-9} , 10^{-10} . The LD₅₀ of the streptomycin treated mice was

$10^{-6.3}$, the LD_{50} of the controls was $10^{-9.3}$. These results are shown in Table 17. It is then apparent that the virulent Sm 1 was immunogenic if the mice were treated with streptomycin in order to hold the infection with the virulent strain in check and allow survival.

Ten of the treated immunized mice and ten normal mice were each injected intravenously with 0.5 ml containing 20 to 40 cells of Sm 1. Two mice of each group were sacrificed at once and 24, 48, 72, and 96 hours after injection. No change in colony type took place during the four days of observation in normal mice. Organisms were recovered from the streptomycin treated mice only after 48 hours and were smooth. Organisms were recovered from the immune mice only at 24 hours and also showed no transformation. This is a somewhat unsatisfactory experiment because of the rapid disappearance of organisms from the immune mice. The dosage greatly affects the survival and multiplication of the challenge strain and therefore this experiment should be repeated with larger dosage.

The behavior of two variants in rats was studied as follows: the variants chosen were 38₃ smooth and completely avirulent for white mice; Sm 1 smooth and highly virulent for mice as shown in the previous experiment. Two groups of nine normal and nine immune rats were used. The latter had recovered from ten LD_{50} doses of Sm A, a smooth virulent variant of Sm. One group of immune and normal rats received 1 ml of 38₃ containing 200 to 400 million organisms. The second group received the same number of Sm 1.

Table VI

The immunogenicity of Sml in mice treated with dihydrostreptomycin

Normal mice (controls)				Mice treated with streptomycin 24,000 units			
Challenge dose	Dead tested	ADD	LD ₅₀	Challenge dose	Dead tested	ADD	LD ₅₀
10 ⁻⁸	9/10	5.0	10 ^{-9.3}	10 ⁻⁶	4/9	7.2	10 ^{-6.3}
10 ⁻⁹	8/10	7.0		10 ⁻⁷	2/10	11.0	
10 ⁻¹⁰	1/10	8.0		10 ⁻¹⁰	3/10	10.3	

Immediately after infection and 24, 48, and 72 hours later, two rats from each group were killed, at 96 hours one rat only was killed. Smear impressions of the heart blood and emulsions of the liver and spleen were cultured and examined for colony type on Snyders and GCBA as described under the mouse experiment.

Three of the normal rats injected with Sm 1 were dead at 48 hours after injection, hence no results are available for the 72 and 96 hour intervals.

The results of this experiment are largely negative in that no organisms were recovered from either the normal or immune rats after injection with 38₃. In the normal rats injected with Sm 1 smooth colonies only were observed after 24 and 48 hours. In the immune rats smooth colonies were observed after 72 hours but no growth was present in the rats killed at 96 hours. No non-smooth Sm colonies appeared in the normal and immune rats in any tissue during this experiment. We have thus been unable to show that non-smooth forms appeared in either normal or immune susceptible animals (mice) or in normal or immune resistant animals (rats).

III. VARIATION STUDIES

C. The multiplication of variants in white mice.

The ability of the several variants to multiply in white mice has been studied extensively in this laboratory in connection with immunogenicity and colony type. The immunogenic strains seem to multiply well even when they are not highly virulent and are always of the smooth type. In this experiment great care was taken in the selection of the strains and all mice were given the same initial dose. The following strains were selected: 38 parent, avirulent smooth and non-smooth; 38₃, avirulent smooth; 38₄, avirulent non-smooth; Jap parent, moderate virulence smooth and non-smooth; Sm parent, virulent smooth and non-smooth; Sm 2, avirulent non-smooth. Mice injected were killed at once and daily thereafter for four days. Spleens were weighed, ground and appropriate serial dilutions were plated. The growth was examined for colony type as before.

The results from the mice injected with 38 were unsatisfactory since no growth occurred even in the inoculum. It is therefore omitted from the table. This organism is more particular about its growth requirements than other strains and lack of growth is a frequent occurrence. It is apparent from Table 18 that the Jap and Sm parent strains grew well and showed no non-smooth colonies. The non-smooth Sm 2 grew also but not as vigorously as the other more virulent strains. It did not show any change from the non-smooth type of colony.

Table 18

The multiplication and morphology of variants during
infection in mice

Time of Sacrifice	Jap (S) inoculum† 4.8×10^3		Sm (S) inoculum 1.7×10^3		Sm 2 (NS) inoculum 8.5×10^2	
	Colony type	Organisms per gram of spleen	Colony type	Organisms per gram of spleen	Colony type	Organisms per gram of spleen
0	0	0	0	0	0	0
24	smooth	2.6×10^5	smooth	6.5×10^6	0	0
48	smooth	1.9×10^6	smooth	4.7×10^8	non-smooth	6.2×10^4
72	smooth	1.7×10^7	smooth	1.7×10^9	non-smooth	1.8×10^5
96	smooth	1.9×10^6	smooth	5.6×10^9	non-smooth	1.8×10^4

† This gives the total number in the inoculum injected.

DISCUSSION

Various immunity reactions in highly susceptible and partially resistant animals have been studied with a view toward understanding the mechanism whereby the animal which has had prior experience with Bacterium tularensis clears his tissues of the living virulent organisms used for challenge.

The properties of the organisms which incite resistance on the part of certain hosts (immunogenicity) and those which account for virulence have also been studied.

In the present report we have prepared polysaccharides from fully virulent and partially virulent immunogenic strains. We have confirmed earlier work in this laboratory that such polysaccharides unlike the pneumococcus polysaccharides are not immunogenic for the white mouse.

We have attempted to isolate immunogenic antigens from the tissues of infected mice and have shown that although the length of life was prolonged by such antigens, a high degree of immunity such as that produced by infection in the white mouse was not induced by these tissue antigens.

A satisfactory protection test has been worked out as well as an evaluation of it. This test will prove valuable in assessing the protective antibodies in whole and fractionated serum and probably also in evaluating vaccines.

We have shown that serum from rabbits which had recovered from infection with immunogenic strains of moderate virulence is more protective than serum from rabbits which had received phenolized

vaccines made from the same strains. Recovered rabbits as well as vaccinated rabbits succumb to a virulent challenge dose in spite of the protective effect shown by their serum. This is one of the examples of the difference with which different species react to infection or vaccination with Bacterium tularense.

Protection tests with rabbit serum which had been fractionated has been used in preliminary experiments in collaboration with the Biochemistry Department.

A series of bactericidal tests on individuals who had been vaccinated with Foshays vaccine or who had recovered from infections acquired in the laboratory showed that whole blood from recovered persons was more bacteriostatic than whole blood from vaccinated persons. The bactericidal or bacteriostatic effect at best is slight, since the blood is never sterilized even when as little as 400 cells per ml of blood is added.

Bactericidal tests on vaccinated and recovered rabbits agreed with the results on human blood but the difference between vaccinated and recovered was more pronounced.

The bacteriostatic effect is evidently not wholly a matter of agglutination since both vaccinated and recovered bloods contained agglutinins.

We report a series of Ascoli tests following Larsons⁽⁴⁾ technique. This promises to be a useful tool and we have shown in this report that the antigen is present most abundantly when the mouse is overwhelmed by infection with virulent strains. It develops in the presence of immunogenic strains such as Jap H, but develops weakly or not at all with strains of low violence. The latter strains produce agglu-

tinins however. It may be present also in the presence of agglutinins but tends to disappear as the animal recovers.

Mice which are challenged after becoming actively immune or after having received immune serum rarely show the Ascoli antigen.

The Ascoli antigen appears not to be immunogenic for white mice. Larson⁽⁴⁾ reported the production of agglutinins in rabbits following the injection of Ascoli antigen.

A series of tests on normal and immune mice have shown that the immune mice removes virulent organisms from the blood stream with great rapidity and concentrates them in the liver. This finding suggests that part of the immunity mechanism in the mouse is due to an efficient phagocytic ability on the part of the fixed tissue cells of the liver. The circulating antibodies of the mouse are only feebly protective, Pannell⁽⁶⁾, and this is true for rabbit and human serum. It is apparent that circulating antibodies as demonstrated by the protective test or by bactericidal tests are not an index of the resistance of the animal to infection. In humans, rabbits, rats and mice agglutinins, protective antibodies and bactericidal antibodies are present after vaccination or after recovery but in the case of mice and rabbits, no solid immunity results after infection with immunogenic strains of moderate virulence, but rabbits do not develop a solid immunity. In man and rats probably a higher degree of immunity results from infection but a fair degree of immunity results from vaccination with killed cultures.

Experiments have been planned to study the combined role of antibodies and phagocytes on immunity in rats, rabbits, mice and humans.

2

A long series of studies on variants of Bacterium tularensis have led to the following accomplishments:

- (a) A technique has been worked out for the recognition of smooth and non-smooth colonies on GCBA plates as well as on Snyders media.
- (b) We have been able to show that our variants fell into 2 groups. Group I is smooth, virulent, immunogenic, toxic, and has the ability to multiply when inoculated into mice in small numbers. Group II is non-smooth, avirulent, non-immunogenic, non-toxic, and multiply poorly in white mice. We are able to select both smooth and non-smooth colonies from avirulent strains but we have no non-smooth strains which are of full virulence.
- (c) Smooth, partially virulent variants induce the formation of high titers of agglutinins in rabbits, and rats. Non-smooth strains induce very low titered agglutinins in rabbits but produce higher titers in rats. Rabbits do not become immune as a result of recovery from living doses of either smooth or non-smooth strains. Rats on the other hand become solidly immune as a result of infection with both types of variants. These results would seem to indicate that the agglutinin present in smooth strains was lacking at least in part in the non-smooth strains. The immunogenic antigen for rats is evidently present in smooth and non-smooth strains. No more immunity to challenge is produced in the rabbit by smooth than by non-smooth strains in spite of the high agglutinin titer.

- (d) Experiments reported briefly here have shown no tendency for smooth strains to dissociate into non-smooth strains in either rats or mice. These studies are being continued.

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APPENDIX

Page

Table I	Results of sterility tests of tissue extracts from infected mice	I
Table II	A. Comparison of the mouse protective effect of immune serum and fractions there of	II
Table III	Clearance tests in passively immunized mice, percentage of the total isolated from tissues	VI
Table IV	Clearance tests in passively immunized mice, number of organisms isolated after intravenous inoculation of rabbit serum	VII
Table V	Clearance tests in passively immunized mice, percentage of the total isolated from tissues	VIII
Table VI	Clearance tests in passively immunized mice, number of organisms isolated after intravenous inoculation of immune rabbit serum and globulin fraction	IX
Table VII	The reciprocal of the agglutinin titer in rabbits injected with variant strains.....	X
Table VIII	The reciprocal of the agglutinin titer in rats injected with variant strains	XI
Table IX	Comparison of antigenic and protective responses following challenge with SmA	XII
Table X	Colonial morphology of organisms obtained from white mice during infection with Sm 1.....	XIII
Table XI	Colonial morphology of organisms obtained from normal and immunized mice after challenge with Sm 1.....	XIV

Table I

Results of sterility tests of tissue extracts from infected mice

Tissue Extract Jap H	Count before centrifuging	Count after centrifuging	Plate count after storage at -50°C			Infectivity after storage at -50°C		
			10 days	21 days	35 days	10 days	21 days	35 days
T ₂₄	6.2 X 10 ⁵	1.3 X 10 ⁴	0	0	0	11/15	3/15	0/15
T ₄₈	3.3 X 10 ⁶	3.2 X 10 ⁴	0	0	0	13/15	14/15	0/15
T ₇₂	4.1 X 10 ⁶	3.5 X 10 ⁴	0	0	0	1/15	0/15	—
T ₉₆	3.1 X 10 ⁶	1.1 X 10 ³	0	0	0	0/15	0/15	---
Tissue Extract Sm			10 days 25 days 45 days			10 days	25 days	45 days 75 days
T ₂₄	2.3 X 10 ³	0 at 10 ⁰	0	0	0	0/15	—	0/15 —
T ₄₈	4.0 X 10 ⁴	0 at 10 ⁰	5	0	0	15/15	14/15	0/13 6/15
T ₇₂	2.9 X 10 ⁴	1.5 X 10 ³	12	0	0	15/15	15/15	0/15 7/15
T ₉₆	1.3 X 10 ⁷	2.3 X 10 ⁴	42	0	0	15/15	15/15	0/15 9/15

-II-

Table II
A comparison of the mouse protective effect of
immune serum and fractions thereof

Rabbit serum or fraction ₁	Serum dilution injected	Challenge strain LD ₅₀ (Ince)	Results					
			Dead Tested	ADD ₂	MPI ₃	MPR ₄	Δ ₅	Σ
Normal 0	10 ⁻¹	2.5 1.5	6/6 2/6	3.5 5.5	28.6 6.1			
Normal 0	10 ⁻²	2.5 1.5	6/6 5/6	4.0 6.6	25.0 11.1			
Normal 0	10 ⁻³	2.5 1.5	5/6 4/6	4.6 6.5	18.1 10.2			
10,240 Jap H Immune whole serum	10 ⁻¹	2.5 1.5	0/6 0/6	— —	C ₇ C	★ ₈ ★	6 2	8
Jap H	10 ⁻²	2.5 1.5	0/6 1/6	— 8.0	C 2.1	★ 5.3	6 4	10
Jap H	10 ⁻³	2.5 1.5	2/6 1/6	8.5 7.0	3.9 2.4	4.6 4.8	3 3	6
5120 Jap H Frac- tion II, III	Undiluted	2.5 1.5	1/6 0/6	8.0 —	2.1 C	No normal un- diluted serum		
Jap H II, III	10 ⁻¹	2.5 1.5	3/6 3/6	7.0 7.3	7.1 6.8	4.0 0.89	3 -1	2
Jap H II, III	10 ⁻²	2.5 1.5	5/6 4/6	5.8 5.5	14.3 12.1	1.7 0.91	1 -1	0
Jap H II, III	10 ⁻³	2.5 1.5	4/6 5/6	7.0 6.4	9.5 13.1	1.9 0.78	1 -1	0
Jap H IV, V, VI 0	Undiluted	2.5 1.5	0/6 0/6	— —	C C	No normal un- diluted serum		
Jap H IV, V, VI	10 ⁻¹	2.5 1.5	0/6 0/6	— —	C C	★ ★	6 2	8
Jap H IV, V, VI	10 ⁻²	2.5 1.5	1/6 0/6	8.0 —	2.1 C	11.5 ★	5 5	10
Jap H IV, V, VI	10 ⁻³	2.5 1.5	2/6 2/3	7.5 6.0	4.4 5.6	4.1 1.8	3 2	5

-III-

Table II (cont.)

Rabbit serum or fraction ₁	Serum dilution injected	Challenge strain ID ₅₀ (Ince)	Results					
			Dead tested	ADD ₂	MPI ₃	MPR ₄	Δ ₅	Σ ₆
1:2560 Jap A Immune whole serum	10 ⁻¹	2.5 1.5	1/6 0/6	4.0 —	4.2 C	6.8 ★	5 2	7
Jap A	10 ⁻²	2.5 1.5	4/6 0/6	6.3 —	10.6 C	2.3 ★	2 5	7
Jap A	10 ⁻³	2.5 1.5	5/6 1/6	5.8 6.0	14.5 2.8	1.2 3.6	0 3	3
1:2560 Jap A Frac- tion II, III	Undiluted	2.5 1.5	1/6 0/6	4.0 —	4.2 C	No normal un- diluted serum		
Jap A II, III	10 ⁻¹	2.5 1.5	0/6 0/6	— —	C C	★ ★	6 2	8
Jap A II, III	10 ⁻²	2.5 1.5	1/6 0/6	6.0 —	2.8 C	8.9 ★	5 5	10
Jap A II, III	10 ⁻³	2.5 1.5	1/6 2/6	7.0 6.0	2.7 5.6	6.7 1.8	4 2	6
Jap A IV, V, VI 0	Undiluted	2.5 1.5	1/6 1/6	7.0 7.0	2.7 2.7	No normal un- diluted serum		
Jap A IV, V, VI	10 ⁻¹	2.5 1.5	5/6 3/6	7.2 7.0	11.3 7.1	2.5 0.86	1 -1	1
Jap A IV, V, VI	10 ⁻²	2.5 1.5	5/6 2/6	5.2 5.5	16.0 6.1	1.6 1.8	1 3	4
Jap A IV, V, VI	10 ⁻³	2.5 1.5	4/6 5/6	6.3 5.4	10.6 11.6	1.6 0.88	1 -1	0
1:320 Jap B Immune whole serum	10 ⁻¹	2.5 1.5	1/6 0/6	9.0 —	1.8 C	15.9 ★	5 2	7
Jap B	10 ⁻²	2.5 1.5	3/6 1/6	7.7 6.0	6.5 2.8	3.8 4.0	3 4	7

-IV-

Table II (cont.)

Rabbit serum or fraction	Serum dilution injected	Challenge strain LD ₅₀ (Ince)	Results					
			Deaf tested	ADD ₂	MPI ₃	MPR ₄	Δ	Σ
Jap B	10 ⁻³	2.5 1.5	4/6 4/6	6.0 6.5	11.1 10.2	1.6 1.0	1 0	1
1:320 Jap B Frac- tion II, III	Undiluted	2.5 1.5	2/6 1/6	9.5 8.0	3.5 2.1	No normal un- diluted serum		
Jap B II, III	10 ⁻¹	2.5 1.5	3/6 1/6	6.3 7.0	7.9 2.4	3.7 2.5	3 1	4
Jap B II, III	10 ⁻²	2.5 1.5	1/6 2/6	5.0 9.5	3.3 3.5	7.6 3.2	5 3	8
Jap B II, III	10 ⁻³	2.5 1.5	4/6 4/6	9.0 9.0	7.4 7.4	2.4 1.4	1 0	1
Jap B IV, V, VI 0	Undiluted	2.5 1.5	1/6 1/6	5.0 7.0	3.2 2.3	No normal un- diluted serum		
Jap B IV, V, VI	10 ⁻¹	2.5 1.5	3/6 1/6	7.0 7.0	7.1 1.6	3.9 3.8	3 1	4
Jap B IV, V, VI	10 ⁻²	2.5 1.5	5/6 6/6	5.4 6.0	15.4 16.7	1.7 0.66	1 -1	0
Jap B IV, V, VI	10 ⁻³	2.5 1.5	3/6 2/6	5.0 6.0	10.0 5.6	1.8 1.8	2 2	4

Legend for Table II

- (1) Immune serum was prepared by the injection of 200 million organisms of the strains indicated.

The serum was fractionated by Cohn's method

Fraction II,III represents gamma and beta globulin

Fraction IV,V,VI represents alpha globulin, albumin and non-protein constituents.

- (2) Average day of death

(3)
$$\text{MPI} = \frac{\% \text{ mortality}}{\text{Average day of death}} = \text{Mouse protective index}$$

(4)
$$\text{MPR} = \frac{\text{MPI normal serum}}{\text{MPI immune serum}} = \text{Mouse protective ratio}$$

- (5) Δ is computed by a comparison between the number of mice dead at a given dilution of organisms plus immune serum and at the same dilution of organisms plus normal serum. The difference in protective power is thus a small whole number. 6/6 normal serum 1:10 + Ince 10^{-4} - 1/6 immune serum 1:10 = Δ_5 + Ince 10^{-4} .

- (6) Σ If the difference between the normal and immune serum at the 10^{-4} dosage is Δ_5 and the difference at the 10^{-5} level is Δ_2 , $\Delta_5 + \Delta_2$ would represent a concrete expression of the protective power of the immune serum at these two test levels or $\Sigma = 7$.

- (7) C = Complete protection

- (8) * = Not computable

Table III

Clearance tests in passively immunized mice,*
percentage of the total isolated from tissues

Category and hour	Per cent of total in Blood	Per cent of total in Liver	Per cent of total in Spleen	Per cent of total in Lung
0				
Normal	96 [†] 55	2.7 36	1.1 6.3	0.3 0.35
Immune	14 3.0	81 87	3.5 3.0	0.20 0.18
6				
Normal	0.02 0.02	85 75	14 23	0.1 0.1
Immune	0.06 0.02	92 72	7.1 27	0.3 0.1
24				
Normal	5.7 0.6	23 63	71 34	0.8 0.7
Immune	0.01 .003	47 63	47 36	5.7 .02

* Immune mice received 0.5 ml of serum (1:10) intravenously followed by Sm 0.5 ml intravenously containing 4 million organisms. The control mice received normal serum. The immune mice were injected with serum pooled from three rabbits which had recovered from an inoculation of 1 ml of Jap H containing 400 million organisms every other day for 3 days. The initial agglutinin titer of the serum was 1:5120.

† The percentages are based on figures given in Table IV and are the per cent of the total from all the tissues in the particular tissue in question.

-VII-

Table IV

Clearance tests in passively immunized mice,★
number of organisms isolated after intravenous inoculation
of rabbit serum.

Time of sampling and category	Agglutinin Titer	Blood	Liver	Spleen	Lung	Total	* of total inoc- ulated
0 hour							
Normal	0	100†	2.87	1.2	0.4	104.4	26
	0	33	22.0	3.8	0.21	60.0	15
Immune	1:32	8.5	49.0	2.1	0.12	59.72	15
	1:64	3.4	96.0	3.3	0.20	110.0	36
6 hour							
Normal	0	0.79	2300	470	3.1	2773.8	
	0	0.25	600	190	1.0	791.0	
Immune	1:16	0.06	835	64	2.7	901.76	
	1:32	0.041	130	50	0.2	180.0	
24 hour							
Normal	0	6400	26,000	79,000	935	111,000	
	0	135	14,000	6700	159	22,000	
Immune	1:8	41	16,000	16,000	1940	34,000	
	1:8	6.8	14,000	8400	46	22,400	

★ Immune mice received 0.5 ml of serum 1:10 intravenously followed by Sm 0.5 ml intravenously containing 4 million organisms. The control mice received normal serum. The immune mice were injected with serum pooled from three rabbits which had recovered from an inoculation of 1 ml of Jap H containing 400 million organisms every other day for 3 days. The initial agglutinin titer was 1:5120.

† The figures express the number of organism per total tissue X 10⁴.

-VIII-

Table V

Clearance tests in passively immunized mice,*
percentage of the total isolated from tissues

Category and hour	Per cent of total in Blood	Per cent of total in Liver	Per cent of total in Spleen	Per cent of total in Lung
0				
Normal	92.6 [†]	6.0	1.2	0.31
Immune	35.3	63.2	0.46	0.61
Fractionated	32.9	65.7	0.71	0.41
6 hour				
Normal	0.07	73.3	26.1	0.12
Immune	0.001	97.8	2.1	0.02
Fractionated	.028	84.7	14.1	0.1
24 hour				
Normal	0.67	44.1	54.5	0.74
Immune	0.04	43.4	56.2	0.18
Fractionated	0.029	39.8	59.7	0.5

* Immune mice received 0.5 ml of serum (1:10) intravenously followed by Sm 0.5 ml intravenously containing 4 million organisms. The control mice received normal serum. The immune mice were injected with serum pooled from three rabbits which had recovered from inoculation of 1 ml of Jap A containing 400 million organisms every other day for three days. The initial agglutinin titer was 1:1280. A third group received fractions of immune serum—Jap A. Jap A serum was produced and fractionated as given in the text.

† The percentages are based on figures given in Table VI and are the per cent of the total from all the tissues in the particular tissue in question.

Table VI

Clearance tests in passively immunized mice,*
number of organisms isolated after intravenous inoculation
of immune rabbit serum and globulin fraction

Time of sampling and category	Agglutinin Titer	Blood	Liver	Spleen	Lung	Total
0 hour						
Normal	0	60. [†]	29.9	0.78	0.2	64.8
Immune	1:32	12.4	22.0	.16	0.22	34.8
Fractionated	0	2.7	.5.4	.058	0.034	8.2
6 hour						
Normal	0	0.34	345.0	125.	0.6	470.9
Immune	1:16	0.023	1225.0	27.	0.25	1252.3
Fractionated	0	0.068	210.0	34.5	0.29	244.9
24 hour						
Normal	0	290.	19,000	23,500	320	43,110
Immune	1:4	3.5	3950	5100	16	9069.5
Fractionated	0	2.5	3400	5100	43	8545.5

* Immune mice received 0.5 ml of serum (1:10) intravenously followed by Sm 0.5 ml intravenously containing 4 million organisms. The control mice received normal serum. The immune mice were injected with serum pooled from three rabbits which had recovered from inoculation of 1 ml of Jap A containing 400 million organisms every other day for three days. The initial agglutinin titer was 1:1280. A third group received fractionated as given in the text.

[†] The figures express the number of organism per total tissue $\times 10^4$

injected with variant strains

[illegible]

Table VIII
The Reciprocal of the Agglutinin Titer in Rats
injected with variant strains

No.	Variant	Days after initial injection								
		2	4	6	10	21	27	29	31	35
1	R ₁	320	160	1280	10240	2560	10240	5120	2560	2560
2	R ₁	0	160	1280	320	320	40960	40960	10240	10240
3	R ₁	320	160	2560	10240	640	1280	2560	2560	1280
4	R ₁	640	40	2560	10240	1280	640	1280	640	640*
5	R ₁	0	160	2560	10240	1280	5120	10240	5120	5120*
Average		24	128	2048	5120	1024	4068	6144	3072	2560
6	R ₂	160	80	20	40	80	160	320	320	160
7	R ₂	80	320	80	160	80	320	320	640	160
8	R ₂	0	80	640	320	40	640	640	320	160
9	R ₂	320	20	640	320	320	160	320	320	20*
10	R ₂	0	20	20	40	1280	640	160	160	160*
Average		56	64	112	128	160	320	320	320	112
11	Jap B	640	80	80	160	640	5120	2560	2560	1280
12	Jap B	320	80	640	320	80	640	1280	640	640
13	Jap B	0	20	640	320	1280	5120	320	2560	1280
14	Jap B	0	320	1280	2560	1280	1280	1280	2560	1280
15	Jap B	0	320	2560	1280	160	640	640	1280	640
Average		48	112	640	576	448	1792	1024	1792	1024
16	Jap H	0	160	10240	5120	20480	640	*	—	—
17	Jap H	0	640	10240	20480	5120	5120	5120	5120	1280
18	Jap H	0	640	10240	20480	2560	10240	5120	5120	20480
19	Jap H	0	1280	10240	10240	*	—	—	—	—
20	Jap H	0	1280	10240	10240	5120	2560	—	1280	640*
Average		0	640	10240	12288	6144	3072	5120	3328	3328
Normal Serum		0	0	0	0	0	0	0	0	0
Antigen Control		0	0	0	0	0	0	0	0	0

* Death either under anesthesia or during bleeding.

Table IX

Comparison of Antigenic and Protective Responses
Following Challenge with Sm A

Variant	Rabbits (1)			Rats (2)		
	Number	Titer (3)	LD (5)	Number	Titer (3)	LD (5)
Ri ₁	100	2560	7.0	1	2560	Survived
	101	1280	5.0	2	10240	Survived
	102	2560	6.0	3	1280	Survived
	103	2560	5.0	4 (4)	640	---
	104	2560	11.0	5 (4)	5120	---
Ri ₂	105	0	8.0	6	160	2.0
	106 (4)	0	---	7	160	Survived
	107	0	4.0	8	160	Survived
	108	0	5.0	9 (4)	20	---
	109	0	4.0	10 (4)	160	---
Jap B	110	0	9.0	11	1280	Survived
	111	0	5.0	12	640	Survived
	112	0	4.0	13	1280	Survived
	113	0	5.0	14	1280	Survived
	114	0	5.0	15	640	Survived
Jap H	115 (4)	320	---	16 (4)	---	---
	116	640	5.0	17	1280	Survived
	117	0	4.0	18	20480	Survived
	118	320	8.0	19 (4)	---	---
	119	320	5.0	20 (4)	640	---
Normals	5 rabbits	0	3.6	4 rats	0	2.0

- (1) Challenge dose: 0.5 ml 10⁻⁷
 (2) Challenge dose: 0.5 ml 10⁻⁸
 (3) Determined 3 days prior to challenge
 (4) Died prior to challenge
 (5) Day of death

Table X

Colonial morphology of organisms obtained from
white mice during infection with Sm 1.

Day after injection	Media used	Normal mice			Streptomycin treated		
		Heart	Liver	Spleen	Heart	Liver	Spleen
Inoculum	GCBA*	100 per cent smooth, buttery, yellow, violet rim					
	Sn	100 per cent smooth, buttery, yellow, violet rim					
0	GCBA	—†	—	—	—	—	—
	Sn	—	—	—	—	—	—
1	GCBA	S††	S	S			
	Sn	S	—	—			
2	GCBA	S	S	S	—	S	S
	Sn	S	S	S	—	S	—
3	GCBA	S	S	S	—	—	—
	Sn	S	S	S	—	—	—
4	GCBA	S	S	S	—	—	—
	Sn	—	S	S	—	—	—

* GCBA = Glucose cysteine blood agar

Sn = Snyders agar

† No recovery of organisms

†† All colonies were smooth

Table XI

Colonial morphology of organisms obtained from normal and immunized mice after challenge with Sm 1

Day after injection	Media used	Normal mice			Immunized mice [*]		
		Heart	Liver	Spleen	Heart	Liver	Spleen
Inoculum	GCBA [†]	100 per cent smooth, buttery, yellow, violet rim					
	Sn	100 per cent smooth, buttery, yellowish, pink centers, violet rims					
0	GCBA	—††	—	—	—	—	—
	Sn	—	—	—	—	—	—
1	GCBA	—	—	—	—	S ^{**}	—
	Sn	—	—	—	—	S	—
2	GCBA	—	—	S	—	—	—
	Sn	—	—	—	—	—	—
3	GCBA	S	S	S	—	—	—
	Sn	S	—	S	—	—	—
4	GCBA	S	S	S	—	—	—
	Sn	—	S	S	—	—	—

* The mice were given an infectious dose of Sm 1 and then treated with streptomycin. This is necessary since an infectious dose of this strain is also a fatal dose.

† GCBA = Glucose cysteine blood agar
Sn = Snyders agar

†† No recovery of organisms

** All colonies were smooth